CYTOTOXIC AND MOLECULAR EFFECTS OF BETEL QUID AND ARECA NUT EXTRACTS ON SELECTED ORAL EPITHELIAL CELL LINES

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by

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LIST OF ABBREVIATIONS

ACKR3	Atypical chemokine receptor 3
AGCC	Affymetrix genechip command console
ANOVA	Analysis of variance
ATCC	American type culture collection
AUC	Area under the curve
BP	Biological process
Bp	Base pair
CC	Cellular component
cDNA	Complementary deoxyribonucleic acid
CI	Confidence interval
CLCA4	Chloride channel accessory 4
CLDN4	Claudin4
cm^2	Square centimetre
CO_2	Carbon dioxide
Cu^{2+}	Cupric Ion
DAPI	4′,6-diamidino-2-phenylindole
DEGs	Differentially expressed genes
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double stranded deoxyribonucleic acid
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate

EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EGLN3	Egl-9 family hypoxia-inducible factor 3
Fe^{2+}	Ferrous Ion
FOSL1	FOS-like antigen 1
G	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCL	Glutamate cysteine ligase
GCLC	Glutamate-cysteine ligase catalytic
GCLM	Glutamate-cysteine ligase modifier subunit
GC-MS	Gas chromatography mass spectrometry
gDNA	Genomic deoxyribonucleic acid
GO	Gene Ontology
GREM1	Gremlin 1
GSH	Glutathione
H	Hour
H_2O	Water
HBEGF	Heparin-binding epidermal growth factor (EGF)-like growth Factor
HIF	Hypoxia-Inducible Factor
HGF	Human gingival fibroblasts
HMOX-1	Heme oxygenase 1
HOCl	Hypochlorous acid
HSC-2	Human oral squamous carcinoma cell line
IARC	International agency for research on cancer
IC50	The half maximal inhibitory concentration
ISO	International organization for standardization

KEGG	Kyoto encyclopedia of genes and genomes
KPNA7	Karyopherin alpha 7
KRT-17	keratin-17
L929	Mouse fibroblast cell line
LB	Lithium borate
MEM	Minimum essential medium
MF	Molecular function
Mg	Milligram
$MgCl_2$	Magnesium chloride
Min	Minute
Ml	Millilitre
Mm	Millimetre
MMP-1	Matrix metallopeptidase 1
MOE1	Mouth-ordinary-epithelium 1
mRNA	Messenger ribonucleic acid
MTT	3-(4,5- dimethyl thiazol- 2- yl) - 2,5-diphenyl tetrazolium bromide
Ng	Nanogram
nM	Nanomolar
OPMDs	Oral potentially malignant disorders
OSCC	Oral squamous cell carcinoma
OSF	Oral submucous fibrosis
PBS	Phosphate buffered saline
PIM1	Pim-1 proto-oncogene
PPARGC1A	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha
QC	Quality control
R^2	Correlation coefficient

RMA	Robust multi-array average
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
ROS	Reactive oxygen species
RPM	Revolutions per minute
Rrna	Ribosomal ribonucleic acid
RT	Retention time
RTK	Receptor tyrosine kinase
RT-qPCR	Real-time reverse transcription-quantitative polymerase chain Reaction
S	Second
SD	Standard deviation
SE	Standard error
SFM	Serum-free medium
SPSS	Statistical package for the social sciences
sscDNA	Single- stranded complementary deoxyribonucleic acid
TAC	Transcriptome analysis console
TdT	Terminal deoxynucleotidyl Transferase
TGF- β	Transformation growth factor beta
TIC	Total Ion Count
TKIs	Tyrosine kinase inhibitors
U/µl	Unit per microliter
UDG	Uracil-DNA glycosylase
V	Voltage
V/V	Volume per volume

VEGF	Vascular endothelial growth factor
WHO	World health organization
Xc−	Glutathione/glutamine antiporter system
$ZnSO_4$	Zinc sulphate
β-actin	Beta actin
µl	Microlitre

KESAN SITOTOKSIK DAN MOLEKULAR DARIPADA EKSTRAK SIRIH PINANG DAN BUAH PINANG KE ATAS SEL EPITELIUM MULUT TERPILIH

ABSTRAK

Tabiat mengunyah sirih adalah meluas di kebanyakan negara di Asia. Kandungan bahan kunyahan sirih pinang termasuk buah pinang, daun sirih, kapur, serta bahan-bahan yang lain seperti tembakau dan esen. Bahan utama sirih pinang adalah buah pinang. Banyak kajian epidemiologi mengaitkan tabiat mengunyah sirih pinang dan buah pinang dengan kanser mulut. Oleh itu, tujuan kajian ini dijalankan adalah untuk mengkaji kesan buah pinang dan sirih pinang ke atas sel *mouth-ordinary-epithelium1* (MOE1) dan sel karsinoma skuamus mulut manusia (HSC-2). Fitokimia ditentukan menggunakan GC-MS. Sel MOE1 dan HSC-2 dirawat menggunakan set kepekatan tinggi (25–100%) dan set kepekatan rendah (0.0122–25%) dan seterusnya, pengasaian MTT dijalankan. Morfologi sel dikenalpasti menerusi mikroskop songsang dan mikroskop berpendarfluor. Selepas itu, data pengekspresan gen adalah tertakluk kepada analisis bioinformatik. Pengesahan data mikrotatasusunan dilakukan dengan cara memilih enam gen dan memprofilkan pengekspresan gen tersebut menerusi tindak balas berantai polimerase transkriptase berbalik masa nyata (RT-qPCR). Arekolina telah dikenalpasti sebagai sebatian kimia utama dalam alkaloid buah pinang, manakala phenol, 2-methoxy-4-(1-propenyl)- pula dikenalpasti sebagai sebatian utama dalam fenol sirih pinang. MOE1 yang dirawat dengan buah pinang menunjukkan penurunan kebolehidupan sel bermula dari kepekatan 0.0244% sehingga 1.56%. Bagaimanapun, pada kepekatan 6.25% dan ke atas, kebolehidupan sel meningkat. Bagi MOE1 yang dirawat dengan sirih pinang, kebolehidupan sel menurun bermula dari kepekatan ekstrak 0.78% (24 jam) atau 3.125% (48 dan 72 jam). Bagaimanapun, kepekatan 50% dan 100%, meningkatkan kebolehidupan sel dengan signifikan. Rawatan buah

pinang ke atas HSC-2 menyebabkan penurunan kebolehidupan sel secara mendadak pada kesemua kepekatan bagi keseluruhan tempoh rawatan. Sirih pinang dengan kepekatan bermula dari 6.25% sehingga 25% mengurangkan kebolehidupan sel HSC-2 dengan signifikan pada semua tempoh rawatan. Berdasarkan data sitotoksik, dua kepekatan dipilih, iaitu 0.0976% ekstrak buah pinang dan 6.25% ekstrak sirih pinang untuk digunakan dalam kajian selanjutnya. Menerusi pemerhatian mikroskopi, didapati ekstrak ini menyebabkan perubahan morfologi yang ketara ke atas titisan sel seperti pengecutan dan pembesaran sel, kondensasi nukleus dan fragmentasi. Data pengekspresan gen mikrotatasusunan menunjukkan bahawa DEG bagi sel MOE1 yang dirawat dengan buah pinang dan sirih pinang berbanding sel kawalan adalah masing-masing sebanyak 3,038 dan 1,985, manakala bagi sel HSC-2 dengan rawatan yang sama masing-masing menunjukkan DEGs sebanyak 4,413 dan 1,110. DEGs yang menunjukkan persamaan dan peningkatan pengekspresan bagi sel yang dirawat dengan buah pinang adalah *CLDN4*, *PIM1*, dan *HBEGF*. Analisis KEGG pula mencadangkan penglibatan gen-gen tersebut dengan beberapa tapak jalan pengisyaratan seperti ErbB. DEG yang menunjukkan persamaan dan peningkatan pengekspresan bagi sel yang dirawat dengan sirih pinang adalah *HMOX-1, GCLM,* dan *EGLN3*. Analisis KEGG pula mencadangkan penglibatan gen-gen tersebut dengan beberapa tapak jalan pengisyaratan seperti feroptosis. Keputusan RT-qPCR bagi gen yang dipilih mengesahkan eksperimen pengekspresan gen mikrotatasusunan yang telah dilakukan. Boleh dirumuskan bahawa ekstrak buah pinang dan sirih pinang menunjukkan kesan yang berbeza ke atas sel epitelium normal dan sel epitelium malignan. Terdapat mekanisma pelindungan molekular pada sel yang dirawat dengan sirih pinang jika dibandingkan dengan sel yang dirawat menggunakan buah pinang.

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ABSTRACT

The habit of betel quid chewing is widely prevalent in many parts of Asia. Betel quid comprises areca nut, betel leaf, lime, and other potential constituents such as tobacco and essences. The main ingredient of betel quid is areca nut. Many epidemiological studies link betel quid and areca nut chewing to oral cancer. Therefore, this study aimed to investigate the effect of areca nut and betel quid on mouth-ordinary-epithelium1 (MOE1) and human oral squamous carcinoma (HSC-2). Phytochemical compounds were identified using GC–MS. MOE1 and HSC-2 cells were treated with high concentrations (25–100%) and low concentrations (0.0122–25%) and subjected to MTT assay. The cell and nuclei morphological changes were observed under inverted phase contrast and fluorescence microscopes. Following microarray analysis, the gene expression data was subjected to bioinformatic analysis. Microarray data was validated by analysing the expression of six selected genes through real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Arecoline was identified as the major chemical compound in areca nut alkaloids, while phenol, 2 methoxy4 (1propenyl) was the major chemical compound in betel quid phenolics. MOE1 treated with areca nut decreased in viability starting from 0.0244% until 1.56%. However, at concentrations 6.25% and above, the cells viability increased. With betel quid treatment, MOE1, cell viability started to decrease at extract concentration of 0.78% (24 hours) and 3.125% (48 and 72 hours). However, at concentration 50% and 100%, the cell viability increased significantly. Areca nut treatment on HSC-2 decreased the cell viability tremendously at all concentrations and treatment time. Betel quid concentration from

6.25% to 25% decreased HSC-2 viability significantly at all treatment duration. Based on the cytotoxicity data, two concentrations were selected, which were 0.0976% for areca nut and 6.25% for betel quid for the subsequent experiments. Microscopy findings indicated that the extracts caused noticeable morphological changes such as cell shrinkage and ballooning, nuclei condensation, and fragmentation. The microarray gene expression analysis revealed that the total number of DEGs in MOE1 treated with areca nut and betel quid compared to controls was 3,038 and 1,985, respectively, while in HSC-2, it was 4,413 and 1,110, respectively. The shared upregulated DEGs of areca nut treatment were *CLDN4, PIM1,* and *HBEGF*. KEGG analysis suggested the genes are associated with few main pathways, including the ErbB signalling pathway. The shared upregulated DEGs for betel quid treatment are *HMOX-1, GCLM,* and *EGLN3*. Its KEGG analysis suggested an association with a few pathways, mainly ferroptosis. RT-qPCR results of selected genes validated the microarray gene expression. In conclusion, areca and betel quid showed different effects on normal and malignant epithelial cells, whereby cell survival mechanism might play an important role in cells treated with betel quid compared to the areca nut treatment.

CHAPTER 1

INTRODUCTION

1.1 Background

Oral diseases are amongst the major public health problems of many societies (Petersen *et al.*, 2005; Ab Halim *et al.*, 2018). Behaviours related to oral health, for example, smoking, betel quid chewing and oral health practices, are strongly related to the occurrence of oral diseases (Anand *et al.*, 2014; Chang *et al.*, 2018; Komar *et al.*, 2018; Quadri *et al.*, 2018).

Oral cancer refers to malignancies that develop in the lip and oral cavity (tongue and mouth) and are one of the most common head and neck cancers (Scully *et al.*, 2008; Shield *et al.*, 2017). Globally, lip and oral cavity cancers comprise the 18th most common malignant neoplasm (Sung *et al.*, 2021). About 90% of all oral cancers are oral squamous cell carcinomas (OSCCs). It is an aggressive malignancy arising from the oral mucosal epithelium, with a high recurrence rate and propensity for lymph node metastasis (Sloan *et al.*, 2017).

In 2020, oral cancer accounted for 377,713 new cancer cases and 177,757 cancer deaths worldwide (Sung *et al.*, 2021). Oral cancer incidence and mortality have shown an increasing trend and are reportedly high in South and Central Asia as well as Melanesia (Miranda-Filho and Bray, 2020; Ren *et al.*, 2020; Sung *et al.*, 2021). Oral cancer is predominantly diagnosed amongst males worldwide and is the leading cause of cancer death in Indian men (Sung *et al.*, 2021). However, in some South-East Asian populations the prevalence may be higher in females (Vatanasapt *et al.*, 2011; Ferlay *et al.*, 2015). The mortality-to-incidence ratio of this cancer in South East Asia is amongst the highest in Asia (Alwan *et al.*, 2010; Ng *et al.*, 2015).

In Malaysia, oral cancer accounted for 1,975 new cases between 2012 and 2016 (Azizah *et al.*, 2019). The reported mortality rate is expected to rise from 253 in 2012 to 336 by 2020, representing a 32.8% increase in mortality rate (Cheong *et al.*,

2017). Although it was not listed among the top ten most common cancer in Malaysia, the incidence is notably high in the Indian ethnic group. Cancer of the oral cavity was reportedly the third and fifth most common malignancy among Indian females and males, respectively (Azizah *et al.*, 2019).

The major risk factors for oral cancer are modifiable, pertaining to lifestyle behaviours, namely excessive alcohol consumption, tobacco smoking and betel quid chewing (Warnakulasuriya, 2010; Winn *et al.*, 2015). In this study, we are interested to investigate the molecular association of betel quid as the risk factor for oral cancer.

Betel quid chewing is practiced by more 600 million people (approximately 10% of the world's population), the majority of whom live in South and South East Asia (Gupta and Warnakulasuriya, 2002; Gupta and Ray, 2004). Betel quid chewing practice is significantly affected by variations in culture and demography across South, Southeast and East Asia (Lee *et al.*, 2011). Additional factors associated with the chewing habit include lower education level, alcohol consumption and tobacco smoking. Although the prevalence of this habit in Sri Lanka, Nepal, Mainland China, and Taiwan is significantly higher in males, the opposite is true for Indonesian and Malaysian populations (Lee *et al.*, 2011). In Malaysia, this habit is more prevalent in rural areas and more frequent among Indians and indigenous people of East Malaysia (Ghani *et al.*, 2011; Lee *et al.*, 2011).

Many epidemiological studies have found that the use of betel quid, with or without tobacco, is strongly linked to oral premalignant lesions and oral cancer in Asian populations, particularly in East and South East Asian populations (IARC, 2004; Lee *et al.*, 2012; Loyha *et al.*, 2012; Muttagi *et al.*, 2012; Gupta *et al.*, 2013; Kampangsri *et al.*, 2013). Based on previous studies, the International Agency for Research on Cancer has classified betel quid with or without tobacco as a Group 1 human carcinogen (IARC, 2004). As such, the higher cancer risk noted among Malaysian Indians and the indigenous people of Sabah and Sarawak may be

attributed to the habit of betel quid chewing (Zain *et al.*, 1997; Zain, 2001; Ghani *et al.*, 2011). Ghani *et al.* (2019) reported that betel quid chewing was the main risk habit among Malaysian oral cancer patients and is the most common habit among Malaysian Indians. In Kelantan, a state with 0.3% Indian population, 22.9% of Malay oral cancer patients reportedly chewed betel quid (Razak *et al.*, 2009).

Betel quid chewing is a habit where the betel quid is placed in between the gum and cheek for extended periods and is consumed through gentle sucking and chewing (Zain *et al.*, 1999; Marya, 2011). Users chew betel quid for the feeling of well-being and euphoria, heightened alertness, focused attention, and it could also diminish hunger and improve digestion (Chu, 2002; Winstock, 2002; Gupta and Ray, 2004; Kyaing *et al.*, 2012).

Betel quid is a masticatory mixture of betel leaf, areca nut and slaked lime (Gupta and Warnakulasuriya, 2002; Gupta and Ray, 2004). Its ingredients and preparation manner tend to vary and may include tobacco and other additives, such as spices, sweeteners, and essences (Gupta and Warnakulasuriya, 2002; Marya, 2011). Areca nut, often a major ingredient of betel quid is the seed of *Areca catechu* fruit (Warnakulasuriya, 2002). It is a species of palm that is locally known as the *Pinang* tree in Malaysia and also cultivated in many other countries including India, Indonesia, Thailand, Singapore, Cambodia, Myanmar, Vietnam, Sri Lanka and Melanesia (IARC, 2004).

Studies on the carcinogenicity of betel quid have begun since the 1960s. The identified harmful ingredient is areca nut which has also been identified as a Group 1 carcinogen (IARC, 2004). Areca nut chemical compounds include polyphenols (flavonols, tannins), alkaloids, carbohydrates, fats, and some crude fibres (Sharan *et al.*, 2012; Gupta and Johnson, 2014; Chen *et al.*, 2017). Variations in areca nut phytochemical compounds may be attributed to different geographical locations, maturity of the nut and different extraction methods (Shwetha *et al.*, 2019; Sari *et al.*, 2020a).

Alkaloids are the most biologically significant areca nut constituent (IARC, 2004a). Various types of alkaloids have been identified namely arecoline, arecaidine, guvacoline and guvacine. Besides alkaloids, other chemical compounds from areca nut such as polyphenols and tannins have been reported to exert carcinogenic and anticarcinogenic effects (Bhide *et al.*, 1979; Jeng *et al.*, 2001; Sharan *et al.*, 2012).

The most abundant alkaloid in areca nut is arecoline, and its carcinogenic effect has been extensively studied (Venkatesh *et al.*, 2018; Gupta *et al.*, 2020; Oliveira *et al.*, 2021). However, there have also been attempts to utilise alkaloid from areca nut as an anticancer agent (Sari *et al.*, 2018; Sari, 2021). However, many studies on arecoline are related to reactive oxygen species (ROS) production during oxidative stress (Shih *et al.*, 2010; Wang *et al.*, 2018). High ROS levels result in cell apoptosis (Circu and Aw, 2010). In addition, arecoline can reduce the antioxidation status (Dasgupta *et al.*, 2006). Cellular glutathione depletion has also been reported in arecoline-induced cytotoxicity (Sundqvist *et al.*, 1989; Chang *et al.*, 2001). In a recent review, ROS-induced DNAdamage, impaired DNA repair with the addition of altered extracellular matrix proteins, enzymes, growth factors, and transcription factors have been suggested to be responsible for the development of oral pathologies attributed to arecoline (Das and Giri, 2020).

Despite the clear epidemiological and experimental association of betel quid chewing with oral cancer, there are substantial gaps in in the understanding of the biological mechanisms behind this relationship (IARC, 2004). One of the reasons is the greater focus on areca nut extract-derived arecoline rather than the betel quid extract. Rasoanaivo *et al.* (2011) reported that crude plant extracts show greater *in vitro* effect than an isolated constituent at the same dose. Thus, the same could be true for the various ingredients that collectively make up a betel quid. Currently, there is a scarcity of information on betel quid molecular mechanism effects on cells

and this is the basis for this study, especially with the availability of high-end molecular technology.

1.2 Justification of the study

As mentioned earlier, previous *in vitro* studies mainly investigated the effects of a single isolated chemical element of betel quid ingredients, such as arecoline, while the information on whole betel quid is lacking. Since the chemical composition of areca nut and betel quid differs, experimental studies on the effects of arecoline, or other single alkaloids alone, will not be able to justify the mechanism of oral cancer. Based on this hypothesis, the current study analysed the microarray gene expression of normal epithelial and carcinoma cell models treated with crude aqueous areca nut and betel quid extracts. Mouth-ordinary-epithelium 1 (MOE1), which retain the characteristics of normal epithelial cells were used as a normal oral epithelial model (Kibe *et al.*, 2011). HSC-2, which is a tongue carcinoma cell line will be used to represent the oral carcinoma model.

The treatment of cell lines with areca nut and betel quid extracts has resulted in multiple alterations of gene expression. The identification of gene expression profiles and suggested pathways following the treatment of cell lines with areca nut and betel quid extracts may provide valuable clues to understand the molecular mechanism underlying the initiation and progression of oral cancer. Therefore, the present study aimed to identify the chemical constituents of crude aqueous areca nut and betel quid extracts. We investigated the cytotoxic effects of both extracts, cell and nuclei morphological changes, and the gene expression profile. The two sets of shared gene profiles for MOE1 and HSC-2 were also analysed, followed with bioinformatics analysis to narrow down the possible pathways that may be involved in oral cancer initiation and development.

The shared genes expressed by both cell lines may serve as potential gene biomarkers for oral cancer related to betel quid aetiology. These biomarkers can be used for future studies such as in diagnostics and targeted therapy.

1.3 Objectives

1.3.1 General Objective

This study was conducted to investigate the effects of crude aqueous areca nut and betel quid filtered extracts on MOE1 and HSC-2 celll lines respectively, based on the cytotoxicity, cell and nuclei morphological changes, gene expression, and bioinformatics analysis.

1.3.2 Specific Objectives

1. To identify the phytochemical compounds present in crude aqueous extracts of areca nut and betel quid using Gas Chromatography-Mass Spectrophotometry (GC-MS) technique.

2. To investigate the cytotoxic effects of crude aqueous areca nut and betel quid filtered extracts on mouse fibroblast (L929), MOE1 and HSC-2 cell lines using MTT assay.

3. To observe the cell and nuclei morphological changes in MOE1 and HSC-2 cell lines after treatment with crude aqueous areca nut and betel quid filtered extracts using inverted light microscope and DAPI staining.

4. To identify, validate and compare the differentially expressed genes (DEGs) between MOE1 and HSC2 cell lines treated with crude aqueous areca nut and betel quid filtered extracts using microarray and RT-qPCR.

5. To analyse and compare the Gene Ontology (GO) terms and enriched Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways of DEGs shared by MOE1 and HSC-2 cell lines treated with crude aqueous areca nut and betel quid filtered extracts using g: Profiler toolset.

1.4 Research questions

1. What phytochemical compounds are present in areca nut and betel quid crude aqueous extracts?

2. Are crude aqueous areca nut and betel quid filtered extracts cytotoxic to L929, MOE1 and HSC-2 cell lines?

3. Do aqueous crude aqueous areca nut and betel quid filtered extracts cause cellular and nuclear morphological changes in MOE1 and HSC-2 cell lines?

4. What are the differences of DEGs between MOE1 and HSC2 cell lines treated with crude aqueous areca nut and betel quid filtered extracts?

5. What are the differences between GO terms and KEGG pathways of shared DEGs in MOE1 and HSC-2 cell lines treated with crude aqueous areca nut and betel quid filtered extracts?

1.5 Research hypothesis

There is a cytotoxic and molecular effects of crude aqueous areca nut and betel quid filtered extracts on MOE1 and HSC-2 cell lines.

CHAPTER 2

LITERATURE REVIEW

2.1 Global prevalence of betel quid chewing

The habit of chewing betel quid is well known in many parts of the world, such as India, China, Pakistan, Indonesia, Sri Lanka, Thailand, Malaysia, Cambodia, Myanmar, Laos,Bangladesh, Taiwan, Papua New Guinea, several Pacific islands as well as among migrant populations in South and Eastern Africa, United Kingdom, North America, and Australia (Gupta and Warnakulasuriya, 2002; Hsiao *et al.*, 2014). According to the Global Adult Tobacco Survey in India (GATS 2), the prevalence of betel quid chewing with tobacco, without tobacco, and areca nut only, varies from 0-39.5%, 0.3-64.9%, and 0.2-22.6%, respectively (TISS, 2018). A study in Karachi, Pakistan, reported that 42.6% of study population were smokeless tobacco and/or betel quid users (Hussain *et al.*, 2017). In Bangladesh, the overall prevalence of betel quid chewing was 33.2%, whereby 17.5% is without tobacco, and 82.5% is with tobacco (Heck *et al.*, 2012). In Nepal, the prevalence of betel quid chewing among 14-18 years old was 30.4% (Wazir *et al.* (2017). The survey among Bhutanese adults showed that 19.7% of the participants were betel quid users (Gurung *et al.*, 2016). In Sri Lanka, the estimated daily betel quid chewing prevalence was 53.8%: 15.7% without tobacco and 47.4% with tobacco (Amarasinghe *et al.* (2018).

A prospective cohort study in Taiwan among young adults showed that 18.7% of male and 0.7% of female subjects were chewers (Lo *et al.*, 2016). In Mainland China, the prevalence of betel quid chewing was 23.9% in men and 1.8% among women (Lee *et al.*, 2012b).

According to an earlier survey in Cambodia, 31.2% of participants were betel quid chewers (6.8% for men and 40.6% for women) (Ikeda *et al.* (1995). Another population-based study conducted among Cambodian adults revealed that 19.7% of

women were betel quid users (Chher *et al.*, 2018). Studies conducted in Singapore and Vietnam reported betel quid chewing prevalence of 6.4% and 6.7%, respectively (Kuek *et al.*, 1990; Reichart and Nguyen, 2008). A cross-sectional survey in Myanmar found that 52% of respondents chewed betel quid on a regular basis. Two hundred and forty of the 284 current betel quid chewers used tobacco to chew their betel quid(Zaw *et al.*, 2016). In Thailand, Chatrchaiwiwatana (2007) study showed that the prevalence of betel quid users was 17%. The study of Lee *et al.* (2012b) in Indonesia revealed that the prevalence of betel quid was12% and 46.8% among men and women, respectively.Another study in Jakarta, Indonesia, found that the prevalence of betel quid chewers was 9.3% (Amtha *et al.*, 2014).

In Papua New Guinea, the prevalence of betel quid chewing varied from 26.8% to 88.4% in different areas (IARC, 2004). Approximately 76.1% of the Palau population chewed betel quid (Ysaol *et al.*, 1996). In a prospective cohort study, Ome-Kaius *et al.* (2015) estimated that of 2700 pregnant women, there were 2249 (83.3%) betel quid users in Madang province in Papua New Guinea. The betel quid chewing prevalence in Guam was 11% (Paulino *et al.*, 2017).

The prevalence of betel quid chewing among South Asia immigrants in Leicester, United Kingdom, reported that betel quid chewing was common among first-generation Asian immigrants, with the highest prevalence amongJains, followed by Muslims and Hindus. In second-generation Asian immigrants, the prevalence of betel quid chewing was the highest among Muslims, followed by Hindusand Jains(Vora *et al.*, 2000). Several other studies also focused on the prevalence of betel quid chewing among Asian immigrants (Farrand and Rowe, 2006; Núñez-de la Mora *et al.*, 2007; Banerjee *et al.*, 2014).

2.2 Malaysian prevalence of betel quid chewing

A nationwide survey was done in the 1990s to acquire baseline data on the prevalence of oral mucosal ulcers(Zain *et al.*, 1995). Gan (1998)studied tobacco use and other oral habits among 431 Bajau women in Sabah. This study revealed that 77% of the women and 4.3% of the men had some type of chewing habit, and betel quid chewing with tobacco (74.2%) was the most prevalent among all types of chewing habits. Tan *et al*. in 2000 conducted a prevalence study on 618 adults from six Malaysian estates. The overall prevalence of betel quid chewing was 119 (19.3%), and the habit was more prevalent among women than men. Ghani *et al.* (2011) performed a study that involved 11,697 adults (with a median age of 42 years) from fourteen states in Malaysia toevaluate the parameters related with the initiation and cessation of betel quid chewing. Betel quid chewers made up 8.2% of the samples. This habit was common among females and the Indians and the indigenous people of Sabah and Sarawak. In the study conducted by the Asian Betel-Quid Consortium, which included 383 men and 620 women in Sabah, Selangor, and Sarawak states of Malaysia, the prevalence of the current betel quid chewers among Malaysian men and women were 9.8% and 29.5%, respectively (Lee *et al.*, 2012b).

2.3 Betel quid chewing and general health

Betel quid chewing habit might impact the oral and general health of chewers (Trivedy *et al.*, 1999). Betel quid is one of the most widely used psychoactive substances worldwide (Boucher and Mannan, 2002; Warnakulasuriya, 2002). The psycho-stimulatory effects that have been reported include heightened alertness, euphoria, and a sense of wellbeing (Chu, 2001; Chu, 2002; Gupta and Ray, 2004; Osborne *et al.*, 2011) due to the effect of betel quid compounds on the central and autonomic nervous systems (Chu, 2001; Winstock, 2002). One of the compounds,

areca nut-derived alkaloids, could bind to gamma-aminobutyric acid receptors in the brain and trigger psychoactive effects (Boucher and Mannan, 2002; Chu, 2002).

The International Agency for Cancer Research classifies betel quid with and without added tobacco as carcinogenic (IARC, 1985, 2012) and can increase the risk of hepatocellular carcinoma, breast cancer, and colorectal polyps (Wang *et al.*, 2003; Kaushal *et al.*, 2010; Chen *et al.*, 2018). Numerous studies have shown that chewing betel quid with or without tobacco is associated with the risk of various systemic diseases, including metabolic and cardiovascular disease (Zhang *et al.*, 2010; Yamada *et al.*, 2013; Khan *et al.*, 2014; Yen *et al.*, 2016). Nicotine and arecoline in tobacco and betel quid can cause dyslipidaemia and hypertension, resulting in cardiovascular disease (Zhang *et al.*, 2010).Some studies found that betel quid consumption was associated with central obesity, diabetes mellitus, cirrhosis, goitre, and hepatic steatosis (Mannan *et al.*, 2000; Tseng, 2010; Paulino *et al.*, 2015; Bleibel and Saleem, 2018; Chu *et al.*, 2018). Betel quid with tobacco use was associated with higher risks for adverse birth outcomes, including preterm birth, low birth weight, and reduced birth length (Yang *et al.*, 2008; Berger *et al.*, 2016).

2.4 Betel quid chewing and oral health

Chronic chewing causes long term exposure of the oral cavity tissue to the chemical constituents of betel quid. This results in deleterious effects on both the hard and soft tissues of the oral cavity (Anand *et al.*, 2014).

2.4.1 Betel quid chewing effects on teeth, periodontium, and bone

Chewing betel quid on a habitual basis is known to be deleterious to the teeth of betel quid chewers due to the hard, fibrous nature of betel quid (Anand *et al.*, 2014; Ilyas *et al.*, 2015). The clinical study of Ilyas *et al.* (2015) revealed that participants who chewed betel quid were more likely to have tooth attrition and missing teeth than those who did not chew betel quid. Dentinal sensitivity was also associated with betel quid chewers, which could be due to enamel loss and exposure of the underlying dentine (Yeh, 1997). The effect of betel quid chewing on the periodontium, i.e., bleeding on probing, probing pocket depth, plaque index and gum recession, was significantly higher among betel quid chewers than non-chewers (Javed *et al.*, 2013; Giri *et al.*, 2014). The deleterious effect of betel quid on periodontium was also shown in many other studies (Hsiao *et al.*, 2015; Wellapuli and Ekanayake, 2017; Rathod *et al.*, 2018; Verma *et al.*, 2019). Hsiao *et al.* (2014) also reported that betel quid chewing significantly increased the radiographic alveolar bone loss. It has been speculated that chewing forces generated during habitual betel quid chewing could increase the deterioration of the temporomandibular joint (Trivedy *et al.*, 2002; Nawaz, 2015).

2.4.2 Betel quid chewing and oral potentially malignant disorders (OPMDs)

OPMDs are clinical presentations in the oral mucosa that carry a risk of developing into oral carcinoma (Reibel *et al.*, 2017). OPMD refers to both the former terminologies "precancerous lesion" and "precancerous conditions" coined in the 70s (WHO, 1973). To date, 12 lesions are listed as OPMDs in the WHO Classification of Head and Neck Tumors 2017, including leukoplakia, erythroleukoplakia, erythroplakia, oral submucous fibrosis (OSF), and oral lichen planus (Reibel *et al.*, 2017). A portion of OPMDs will undergo malignant transformation, although the prevalence varies across the spectrum of diseases

(Warnakulasuriya *et al.*, 2020). Oral erythroplakia has been identified as the one with the highest malignant transformation rates (Villa *et al.*, 2011). The pathogenesis of OSF in relation to areca nut, a constituent of betel quid, is more frequently discussed in the literature than other OPMDs.

In Asia, the prevalence of OPMD was estimated to be 1.7% to 11.7% in western India (Napier and Speight, 2008), 4.4% to 12.7% in southern Taiwan (Chung *et al.*, 2005; Yang *et al.*, 2010), 0.1% to 4.7% in the Hunan province of Mainland China (Zhang and Reichart, 2007), 6.7% in central Sri Lanka (Ariyawardana *et al.*, 2007), and 1.4% in Malaysia (Zain *et al.*, 1997b).OSF has also frequently been reported in India, Southeast Asia, and Britain among the Asian immigrant populations of Britain and America (Arakeri *et al.*, 2017).

The evidence of the role of betel quid use, with or without tobacco, increasing the risk of the development of OSF is based on many previous case reports, prospective cohort studies as well as several case-control and cross-sectional studies that have been conducted in many countries, including India, Pakistan, Sri Lanka, Taiwan, and Malaysia. The Taiwanese studies have indicated a significant association between OSF and the chewing of betel quid without tobacco (Yang *et al.*, 2001; Lee *et al.*, 2003; Chung *et al.*, 2005; Yang *et al.*, 2005; Chen *et al.*, 2006b; Yen *et al.*, 2007). Chewing betel quid with tobacco was associated with OSF in many studies from Sri Lanka, India, and Pakistan (Maher *et al.*, 1994; Ariyawardana *et al.*, 2006; Srivastava *et al.*, 2019; Tejasvi *et al.*, 2019). In Malaysia, the Indians and indigenous people of East Malaysia had the highest prevalence of precancerous lesions, including leukoplakia, erythroplakia, submucous fibrosis and lichen planus and may be attributed to betel quid consumption (Zain *et al.*,1997b).

OSF is an insidious, chronic disease that affects any part of the oral cavity and sometimes the pharynx (Arora *et al.*, 2014). Its pathogenesis is linked mainly to alkaloids from the areca nut, resulting in abnormal collagen synthesis and degradation (Ahmad *et al.*, 2006). This results in progressive fibrosis of the

submucosae with epithelial atrophy, leading to stiffness of the oral mucosae, trismus, and inability to eat (Pillai *et al.*, 1992).

2.4.3 Betel quid chewing and oral cancer

Studies in Asian regions and Melanesia have reported the high risk of developing oral cancer and chewing betel quid with or without tobacco (Ko *et al.*, 1995; Thomas *et al.*, 2007; Song *et al.*, 2015; Uddin, 2017; Azhar *et al.*, 2018). Previous studies have also shown a similar association between betel quid chewing and oral cancer in Malaysia, and the habit is more commonly found in Indian patients and the indigenous people of Sabah and Sarawak (Ahluwalia and Duguid 1966; Ng *et al.*,1986; MOH, 2002). Cancer in these patients typically involved the buccal and vestibular mucosa, which corresponds to the site of quid placement.Oral cancer deaths in Malaysia reached 1,140 in 2017, accounting for 0.82% of all deaths, according to WHO data. As mentioned above, the epidemiological studies have indicated an association between betel quid chewing with oral precancerous lesions and oral cancer. Therefore, many previous studies were conducted to identify the phytochemical compounds of betel quid and areca nut (Salutan and Billacura, 2009; Amudhan *et al.*, 2012; Sari *et al.*, 2020b). However, since scientists have already determined the carcinogenic potential of alkaloids derived from areca nut, much emphasis has been placed on these alkaloids in many studies.

2.5 Betel quid composition

Quid is defined as a mixture of substances placed in the mouth that typically contains at least one of the two basic constituents, areca nut or tobacco, in its raw or manufactured or processed form(Zain *et al.*, 1997a).Betel quid is often made up of areca nut, betel leaf, slaked lime, and occasionally tobacco (IARC, 2004). Additional ingredients, particularly spices such as cloves, turmeric, cardamom, saffron, aniseed, mustard, or sweeteners, are added to suit local tastes(Winstock, 2002;

IARC, 2004). The ingredients will be placed on the betel leaf, folded, and the quid is ready to be chewed [Figure 2.1 (A)].

2.5.1 Areca nut

Areca nut is the seed of the *Areca catechu* fruit of the oriental palm. It is typically produced in the form of slices for betel quid (Figure 2.1: B) (Warnakulasuriya *et al.*, 2002; Blank *et al.*, 2008a; Blank *et al.*, 2008b). The Areca catechu fruit is oval in shape with a pointed apex (Figure 2.1: C).When the fruit is unripe, it is green; when it is ripe, it isorange-yellow(IARC, 2004). The seed has a distinctive astringent and somewhat bitter flavour and can be ingested at various stages of maturation(IARC, 2004).The nut can be consumed fresh, dried, or cured in the sun, oven, or roaster (IARC, 2004).

2.5.2 Betel leaf

The betel leaf is the betel vine's leaf (Figure 2.1: D). Betel vine belongs to the family of *Piperaceae,* which is believed to have originated from Malaysia (Jaiswal *et al.*, 2014). It is widely consumed as a condiment in Africa and Asia. Betel leaves are used to encase the ingredients in a betel quid.

2.5.3 Slaked lime

On betel leaves, slaked lime is frequently smeared (Figure 2.1: E). It is derived from burned and crushed seashells or corals(IARC, 2004; Sazwi *et al.*, 2013). However, slake lime is quarried from limestone in non-coastal areas. Slaked lime is sold in Asian markets as a paste that is mixed with water(IARC, 2004).

2.5.4 Betel inflorescence

Apart from the leaf, other parts of the betel vine, such as the stem, inflorescence or catkins, are consumed while chewing betel quid (IARC, 2004). Consumption of inflorescence is widespread in some countries, including Taiwan and Melanesia, where it is added to quid to impart an aromatic flavour (IARC, 2004).

2.5.5 Tobacco

Tobacco is frequently added to the quid mixture in the form of coarsely chopped leaves (Figure 2.1: F). It is made without further processing from sun-dried and partially fermented *Nicotiana rustica* and *Nicotiana tabacum*(IARC, 2004). Occasionally, tobacco is powdered, blended with molasses, or boiled prior to use (IARC, 2004).

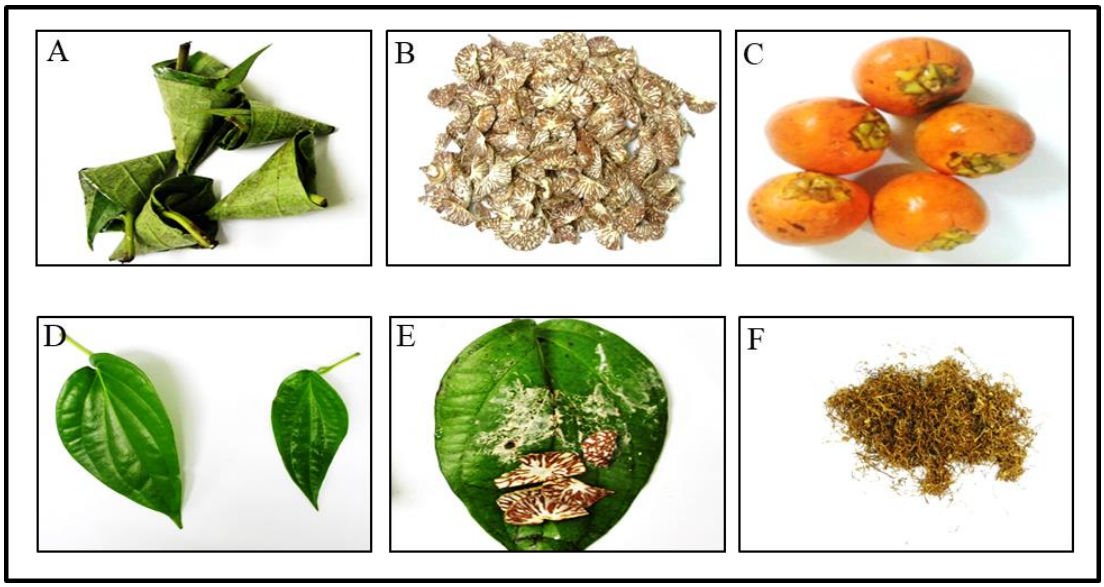

Figure 2.1 Betel quid and its ingredients. (A): Ready to chew quid, (B): Dried areca nut flakes, (C): Ripe areca catechu fruits, (D): Betel leaves, (E): Slaked lime and areca nut slices placed on betel leaf, and (F): Dried, shredded tobacco leaves.

2.6 Betel quid preparation and consumption

The ingredients and methods used to prepare betel quid varies, based on geographical regions (Gupta and Ray, 2004). In India, the use of betel quid can be classified into traditional and modern forms. The traditional form of betel quid is called paan, and the typical components of paan are lime, areca nut, tobacco, spices, and betel leaf (Shah *et al.*, 2012). The paan industry became big when paan was developed into ready-to-eat products, in small sachets, known as gutkha(Shah *et al.*, 2012). The ingredients of gutka are lime, areca nut, tobacco, spices, and catechu (Shah *et al.*, 2012). Catechu is a reddish-brown substance derived from the *Acacia catechu* tree's heartwood (Muir and Kirk, 1960).

In Sri Lanka, tobacco-added betel quid is more commonly used than the tobacco-free betel quid form (Lee *et al.*, 2012a). Chewing betel quid is also popular in southern China, particularly inHainan Island and Hunan Province(Tang *et al.*, 1997; Liu *et al.*, 2015). In Hainan Island, the betel quid is a combination of fresh areca nut, lime and betel leaf (Tang *et al.*, 1997). In Mainland China, betel quid is industrially packaged and sold in small bags. The final products use half-dried fruits, including the husk with different flavoured substances and tobacco is never added (Zhang and Reichart, 2007).

The hill tribes of Cambodia, Myanmar, Thailand, and Laos infuse their betel quid with cloves, cinnamon, and the roots of certain indigenous plants (Awang, 1983). In Taiwan, unripe areca nuts are frequently chewed with slaked lime; betel quid may contain betel leaf or betel inflorescence but not tobacco (Wen *et al.*, 2005). In Cambodia, the majority of users would combine their betel quid with tobacco (Singh *et al.*, 2012). A betel quid is a mixture of betel leaf, areca nut, lime, and tobacco found in Indonesia (Amtha *et al.*, 2014). The areca nut is typically chewed in Papua New Guinea along with betel inflorescence and lime (Thomas and MacLennan, 1992).

Generally, the areca nut is used as one of the additives in betel quid, but it can be chewed alone without any other ingredients. Nonetheless, there are limited available data on the use of areca nut without any other ingredients. However, the Chamorros people in Micronesia would swallow the masticated areca nut (with or without betel leaf), and they do not add slaked lime or tobacco (Paulino *et al.*, 2011).

The traditional quid in Malaysia is made with slaked lime, areca nut, and flavouring ingredients wrapped in a betel leaf(IARC, 2004). In 1970, Chin and Lee reported that the betel quid essential ingredients in West Malaysia contained sliced dried areca nut, slaked lime, and betel leaf. The betel leaf may be young or mature, while the areca nut is sometimes consumed fresh. The additional ingredient could be tobacco and gambir, with the former being more frequently used (Tan *et al.*, 2000). Gambir is made by boiling the leaves and bark of *Uncaria gambir*, a member of the *Rubiaceae* family(Sazwi *et al.*, 2013).

2.7 Phytochemical compounds of betel quid and areca nut

The major biochemical compounds in areca nut are alkaloids, fatty acids, minerals and tannins (Zhang *et al.*, 2008; Vanimakhal and Ezhilarasi, 2016). The four major alkaloids in areca nut are arecoline, arecaidine, guvacoline and guvacine (Garg *et al.*, 2014). Areca nut contains many fatty acids includingmyristic, stearic, decanoic, oleic, dodecenoic, tetradecenoic and hexadecenoic (Jaiswal *et al.*, 2011). The minerals in the areca nut include calcium, phosphorus, and iron (Raghavan and Baruah, 1958). Alkaloids, especially arecoline, are frequently associated with the areca nut's carcinogenic risk (IARC, 2004).

Sari *et al.* (2020a) reported that betel quid mixture of slaked lime, betel leaf, and areca nut contained alkaloids and phenolic compounds. Phenolic chemicals in betel leaf act as anti-mutagenic agents, lending the betel leaf a protective role against the areca nut's toxic alkaloids (Jeng *et al.*, 1994). Betel leaf extract inhibits the mutagenic action of standard mutagens like benzo[a]pyrene and

dimethylbenz[a]anthracene (Bhide *et al*., 1991). The anticancer, antioxidant, and superoxide radical scavenging properties of phenolic compounds have contributed to their beneficial effects (Djeridane *et al.*, 2006; Sazwi *et al.*, 2013). The deleterious effects of various areca nut components, including arecoline, have been studied through *in vitro* assays (Peng *et al.*, 2015; Chuerduangphui *et al.*, 2018).

2.7.1 Phytochemical analysis using GC-MS

GC-MS is a hyphenated technique that is extremely compatible and is the most frequently used technique for quantification and identification(Padma *et al.*, 2019). By interpreting and comparing the spectra to those of known organic compounds in a complex mixture, the unknown organic compounds can be identified (Yonzone *et al.*, 2012). GC-MS instrument consists of two main components, each with different functions.

Gas chromatography (GC) is used to separate distinct compounds in a complicated mixture, whilst mass spectrometry (MS) is used to identify and quantify the active compound's specific structural information (Iordache *et al.*, 2009). The mobile phase of GC consists of a gas carrier, whereas the stationary phase is an insert solid layer that supports the inside of a tube known as a column(Hussain and Maqbool, 2014). During sample travels in the column, the separation of distinct chemical characteristics between different molecules in a mixture of their relative affinity usually happens in the stationary phase (Hussain and Maqbool, 2014).

The selectivity of capillary columns is influenced by column dimensions and factors such as length, diameter, and film thickness(Sahil *et al.*, 2011).The molecules are held in the column and elute at different times, which is referred to as the retention time (RT).The mass spectrometer downstream will then be able to catch, ionise, accelerate, and identify the ionized molecules independently (Hussain and Maqbool, 2014). By breaking each molecule into ionised fragments and identifying these fragments based on their mass-to-change ratio (m/z), the identified and quantified chemicals are analysed with MS (Kanthal *et al.*, 2014). To identify the

phytochemical substances, the peaks in the chromatogram of the sample examined are compared to the spectrum database of known components contained in the GC-MS library(Thomas *et al.*, 2013). Figure 2.2 depicts a schematic diagram of the GC-MS system.

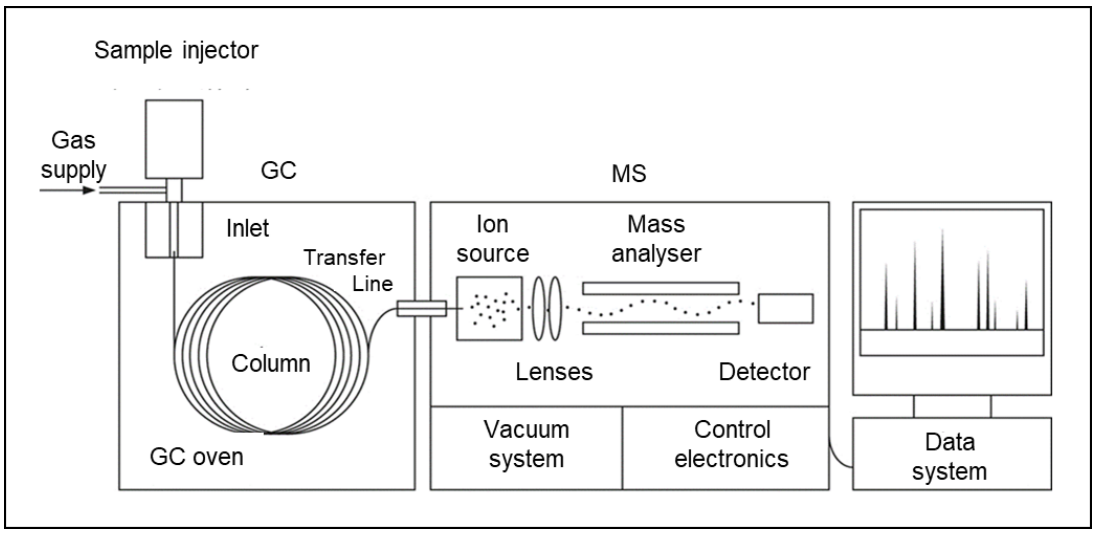

Figure 2.2 Schematic diagram of GC-MS system. [Adapted from Sutherland (2018)].

2.8 Cellular and molecular effects of betel quid and areca nut

Previous studies have reported that betel quid and areca nut can cause oxidative stress (Lu *et al.*, 2010; Chan *et al.*, 2019). The stress results from reactive oxygen species (ROS) production due to selected chemical compounds derived from betel quid or areca nut (Chan *et al.*, 2019).

2.8.1 ROS and oxidative stress induction

Chemical and metabolic cellular activity convert oxygen to ROS production (Zhang *et al.*, 2011), which contribute to reduction-oxidation (redox) state. ROS are by-products of normal cellular metabolism that play an important role in signalling pathways in response to intracellular and extracellular environmental changes (Jabs, 1999). Most ROS are generated in cells by the mitochondrial respiratory chain (Poyton *et al.*, 2009). ROS plays a role in normal cellular physiology functions such as cell proliferation and differentiation in redox homeostasis. However, in the state of redox imbalance (for example - high ROS level), senescence, apoptosis, and cell death may occur (Ye *et al.*, 2015). During exposure to environmental stress, such as ultraviolet radiation, heat and ionising radiation, intracellular ROS levels could increase dramatically (Wood *et al.*, 2003; Finkel, 2011). ROS readily react with proteins, lipids, nucleic acids, and carbohydrates, causing oxidative stress and substantial damage to cell components (Cross *et al.*, 1987; Conner and Grisham, 1996; Brieger *et al.*, 2012).

Oxidative stress refers to a pathologic state arising from an imbalance between cellular ROS and cell ability to detoxify them and affecting several signalling pathways (Uttara *et al.*, 2009). It can also be associated with reactive nitrogen species (RNS) (Ye *et al.*, 2015).

ROS are tiny compounds formed from oxygen molecules, such as superoxide ($O2^{\cdot}$), hydroxyl ($^{\cdot}OH$), peroxyl ($RO_{\mathbf{2}}$.), and alkoxyl (RO^{-}), as well as non-radicals such as hypochlorous acid (HOCl), ozone (O_3), singlet oxygen ($^1O_2$), and

hydrogen peroxide (H_2O_2)(Ozcan and Ogun, 2015). These non-radicals are oxidizing agents that convert rapidly to radicals. Nitric oxide (NO), peroxynitrite (ONOO), and nitrogen dioxide (NO_2) are nitrogen-containing oxidants (Klebanoff, 1980; Bedard and Krause, 2007).

The ingredients combined in betel quid produce ROS during betel quid chewing (Nair *et al.*, 1987; Thomas and MacLennan, 1992; Chen *et al.,* 2006a). Liu *et al.* (1996) reported that ripe areca nut-induced ROS resulted in higher oxidative damage to cells than young areca nut. In response to oxidative stress induced by areca nut, many studies highlighted the upregulation of many genes via different biological processes and pathways, including hypoxia-inducible factor-1(HIF-1), autophagy, cell cycle arrests, vascular endothelial growth factor (VEGF) and glutathione (Smith and Fornace, 1996; Lu *et al.*, 2010; Ji *et al.*, 2014; Kwon *et al.*, 2019).

ROS is directly involved in the tumour initiation process through genotoxicity (Nair *et al.*, 1990; Sundqvist and Grafström, 1992). Because of mitochondrial malfunction, altered metabolism, and genetic abnormalities, ROS generation in cancer cells is considerably elevated, resulting in enormous levels of oxidized protein, DNA, and lipids (Demple and Harrison, 1994; Visconti and Grieco, 2009). As a result, cancer cells have higher quantities of ROS-scavenging molecules as an adaptive response (Samanta *et al.*, 2016; Ciccarese and Ciminale, 2017).

In addition, Khan *et al.* (2015) highlighted that areca nut extracts had induced ROS in human keratinocytes. Oral KB carcinoma cells exposed to areca nut extract can also produce ROS (Chang *et al.*, 2001b). A similar reaction is also shown by OSCC when treated with areca nut (Lu *et al.*, 2010). Induced biosynthesis of glutathione (GSH) is a protective measure for the survival of cancer cells. GSH is an antioxidant that acts as a free radical scavenger and a detoxifying agent in cells (Traverso et al., 2013; Haenen and Bast, 2014; Nimse and Pal, 2015).

2.8.2 Cytotoxicity effects of betel quid and areca nut

2.8.2(a) Cytotoxicity in general

Cytotoxicity is the toxic effect of material on cells that either kills the cells or alter their metabolism (Freshney, 2010). The macromolecular synthesis is disrupted by a series of molecular processes, causing specific functional and structural damage to the cells (Anderson *et al.*, 1993). Hence, cytotoxicity assays are necessary to identify the cytotoxic nature of test substances (McGaw *et al.*, 2014). The identification of common cytotoxicity assays is conducted based on the measurements of metabolic activity that indicate the cytotoxic nature of extracts of plant and its purified compounds (Bunel *et al.*, 2014; McGaw *et al.*, 2014). *In vitro* cytotoxicity tests define basal cytotoxicity, such as the intrinsic ability of a particular test substance to cause cell death. The test substances are considered cytotoxic if they interfere with the cell attachments, alter the cell morphology, affect cell growth and cause cell death (Horvath, 1980). This assay is important in determining the concentration range for further *in vitro* testing such as genotoxicity, induction of mutations or programmed cell death. The data can also be utilised in determining acute systemic toxicity (Eisenbrand *et al.*, 2002).

To assess cell viability, various types of cytotoxicity tests are performed. The type of the test drug, the expected response, and the target cell all influence which viability assay is best for cytotoxicity testing (Freshney, 2010; McGaw *et al.*, 2014).

To determine harmful effects of plant extracts and pure plant chemicals, conventional cytotoxicity of cell viability assays is identified based on metabolic activity measurements (Bunel *et al.*, 2014; McGaw *et al.*, 2014). Tetrazolium and resazurin tests are two types of metabolism reductase viability assays (Bunel *et al.*, 2014; McGaw *et al.*, 2014).

2.8.2(b) Betel quid and areca nut cytotoxicity

Report on betel quid effects can be found since 1960, where besides being cytotoxic, it is also genotoxic (Sen *et al.*, 1989; Jeng *et al.*, 1994). Because of this,