ELUCIDATION OF THE MECHANISM INVOLVED IN THE PROLIFERATION AND MIGRATION ACTIVITY OF *CYPERUS ESCULENTUS* LATIVUM IN MDA-MB-231 AND MCF7 BREAST CANCER CELL LINES

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by

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LIST OF ABBREVIATIONS

µg	Micrograms
µL	Microliter
APS	Persulphate
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine triphosphate
BRCA1	Breast cancer susceptibility gene 1
BRCA2	Breast cancer susceptibility gene 2
BSA	Bovine serum albumin
CCNA2	Protein-coding gene for Cyclin A2
CCND1	Protein-coding gene for Cyclin D1
CCNE1	Protein-coding gene for Cyclin E1
CDC25a	Protein-coding gene for Cdc25a
CDK1	Cyclin dependent-kinase 1
CDK2	Cyclin dependent-kinase 2
CDK4	Cyclin dependent-kinase 4
CDK6	Cyclin dependent-kinase 6
CDKN1A	Protein-coding gene for p21
CDKN2A	Protein-coding gene for p16 and p14
CDKN2C	Protein-coding gene for p18
cDNA	Complementary deoxyribose nucleic acid
CM	Complete medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
ER	Estrogen receptor
ERK	Extracellular signal -regulated protein kinase
FBS	Fetal Bovine Serum

$FeCl_3$	Ferric chloride
g	Grams
GSK3β	Glycogen synthase kinase 3β
GTP	Guanosine triphosphate
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
HER2	Human epidermal growth factor receptor 2
JNK	c-Jun NH_2-terminal kinase
LIMK1	LIM domain kinase 1
MAPK	Mitogen-activated protein kinase
min	Minutes
mg	Milligrams
MLC1	Megalencephalic leukoencephalopathy with subcortical cysts 1
MTT	3-[4, 5-dimethyl thiazol-2-yl] 2, 5-diphenyl tetrazolium bromide
Na_2CO_3	Sodium Carbonate
NH3	Ammonia solution
PAK1	Serine/threonine-protein kinase PAK 1
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PR	Progesterone receptor
qPCR	Quantitative Real-time Polymerase Chain Reaction
R^2	correlation coefficient
RNA	Ribonucleic acid
ROCK1	Rho-associated protein kinase 1
SDS	Sodium Sodecyl Sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with 0.1% Tween-20
TGF	Transforming growth factor
TNBC	Triple-negative breast cancer
TP53	Protein-coding gene for p53
UV	Ultraviolet
v/v	Volume per volume

VA	cis-Vaccenic acid
w/v	Weight per volume
WT	Wild type
X*g*	Centrifuge force

KAJIAN TENTANG AKTIVITI EKSTRAK *CYPERUS ESCULENTUS* LATIVUM PADA MEKANISMA PERTUMBUHAN DAN PENGHIJRAHAN DALAM SEL KANSER MDA-MB-231 DAN MCF7

ABSTRAK

Cyperus esculentus L., lazimnya digunakan sebagai tepung dan minyak dalam industri makanan. Kebanyakannya dimakan mentah sebagai makanan ringan kerana berperisa susu manis. Minyak daripada *C. esculentus* mengandungi beta-sitosterol, asid oleik dan quercetin, yang merupakan kumpulan molekul yang mempunyai sifat antikanser pada pelbagai sel kanser. Kajian ini bertujuan untuk mengkaji kesan antiproliferatif ekstrak etanol dan air *C. esculentus* pada sel kanser MDA-MB-231 dan MCF7. Selain itu, kesan ekstrak pada kitaran sel dan aktiviti penghijrahan sel untuk kedua-dua sel kanser telah disiasat. Mekanisme yang terlibat dalam aktiviti ekstrak tersebut juga dielusidasikan. Kaedah MTT dijalankan untuk memeriksa kesan ekstrak ke atas viabiliti kedua-dua sel kanser, manakala flow cytometry digunakan untuk menilai perkembangan kitaran sel. Selain itu, assay penyembuhan luka dan matrigel telah dijalankan untuk memeriksa aktiviti penghijrahan sel, manakala qPCR dan Western blot dilaksanakan untuk menilai ekspresi gen dan protein. Keputusan menunjukkan bahawa ekstrak *C. esculentus* hanya mengurangkan viabiliti sel kepada 60 % pada rawatan 24 rawatan dan 80 % pada rawatan 48 jam di MDA-MB-231 dan MCF7, masing-masing. Tempoh rawatan yang berlanjutan dan peningkatan kepekatan ekstrak tidak menjejaskan viabiliti sel lagi. Selain itu, ekstrak menghentikan kitaran sel di G0/G1 untuk MCF7, manakala dalam MDA-MB-231, kedua-dua tahap S dan G2/M penghentian kitaran sel telah diperhatikan. Untuk kajian ekspresi mRNA, cyclins yang berkaitan dengan kitaran sel (CCNA2, CCNE2) dan kinase yang

bergantung kepada cyclin (CDK1, CDK2, CDK6) tidak dikesan dalam MDA-MB-231, sedangkan hanya CCNE2 dan CDK4 tidak hadir dalam MCF7. Daripada semua inhibitor kinase yang bergantung kepada cyclin yang disiasat, hanya gen p53 yang hadir dan ekspresinya meningkat dalam MDA-MB-231; manakala ekspresi gen CDKN1A, CDKN2A, dan CDKN2C didapati meningkat dalam MCF7. Untuk kajian protein, p44/42-MAPK dan c-Myc dikesan meningkat dalam MDA-MB-231. Dalam MCF7, protein c-Jun diinduksi tetapi cyclin D1 dihalang. RhoA dihalang dalam kedua-dua sel kanser yang dirawat, manakala Rac1/2/3 diinduksi dalam MCF7. Perencatan RhoC hanya diperhatikan dalam rawatan Ym di kedua-dua sel kanser. Ekspresi CDKN1A, CDKN2A dan CDKN2C yang tinggi bertanggungjawab untuk penghentian fasa G0/G1 yang diperhatikan dalam MCF7, manakala penghentian di tahap S dan G2/M dalam MDA-MB-231 adalah disebabkan oleh induksi p53. Ekspresi gen dan protein juga menunjukkan bahawa proliferasi MDA-MB-231 dan MCF7 serta penghentian kitaran sel telah dihalang melalui laluan isyarat MAPK dan c-Jun. Di samping itu, kesan melalui p41/p42 MAPK dan c-Jun telah dimulakan melalui modulasi cyclin D1 dan c-Myc dalam MDA-MB-231 dan MCF7. Tambahan pula, ekstrak tersebut juga menurunkan motiliti MDA-MB-231 dan MCF7 melalui modulasi RhoA, RhoC dan Rac1/2/3. Kesan ekstrak *C. esculentus* didapati bergantung kepada jenis sel. Oleh itu, *C. esculentus* berpotensi untuk berfungsi sebagai sumber baru dalam membangunkan agen-agen novel terhadap pelbagai jenis kanser payudara.

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ABSTRACT

Cyperus esculentus L., is a tuber of family Cyperaceae which produces rhizomes from the base. It is used mostly as flour and oil in the food industry. In Ghana, it is mostly consumed raw as a snack because of its milky and sweet taste. The oil contains beta-sitosterol, oleic acid and quercetin, which are the compounds found to have anticancer property on various cancer cell lines. This research aimed to investigate the antiproliferative effect of the ethanolic and aqueous extracts of C. *esculentus* on MDA-MB-231 and MCF7 breast cancer cell lines. Also, the effects of the extracts on cell cycle progression and cell migration activity of both cell lines were studied. The possible mechanisms involved in the observed activity of the extract were also elucidated. MTT assay was run to check the extract effect on the viability of both cell lines, whereas flow cytometry was used to assess the cell cycle progression. Wound healing and Matrigel assays were run to check the cell migration activity, whereas qPCR and western blotting were run for gene and protein expressions, respectively. The results show that C. *esculentus* extracts only reduced the cell viability to 60 % at 24 hours and 80 % at 48 hours of treatment in MDA-MB-231 and MCF7, respectively. Prolong treatment and increase concentration did not affect the cell viability further. Also, the extracts induced G0/G1 cell cycle arrest in MCF7, but in MDA-MB-231, both S and G2/M phases cell cycle arrests were observed. For mRNA expression study, the cell cycle related cyclins (CCNA2, CCNE2) and cyclin-

dependent kinases (CDK1, CDK2, CDK6) were not detected in MDA-MB-231, whereas only CCNE2 and CDK4 were absent in MCF7. Out of all the cyclin-dependent kinases inhibitors investigated, only p53 gene was present and upregulated in MDA-MB-231; while CDKN1A, CDKN2A, and CDKN2C genes were found to be upregulated in MCF7. For protein study, the p44/42-MAPK and c-Myc were detected to be overexpressed in MDA-MB-231. In MCF7, c-Jun protein was induced but cyclin D1 was inhibited. RhoA was inhibited in both treated cell lines, while Rac1/2/3 was induced only in MCF7. RhoC inhibition was observed only in the Ym-treatment in both cell lines. The overexpression of CDKN1A, CDKN2A and CDKN2C is responsible for the G0/G1 phase arrest observed in MCF7, while the S and G2/M phases arrest in MDA-MB-231 is due to the induction of p53. The gene and protein expressions also showed that MDA-MB-231 and MCF7 proliferation and its cell cycle arrest were inhibited via MAPK and c-Jun signaling pathways. Also, the effect through p44/p42 MAPK and c-Jun was initiated via the down- and up-regulation of cyclin D1 and c-Myc in MDA-MB-231 and MCF7, respectively. In addition, the extracts suppress MDA-MB-231 and MCF7 motility through the modulation of RhoA, RhoC, and Rac1/2/3. C. *esculentus* extract-induced pathways are found to be cell type dependent. Thus, C. *esculentus* might serve as a potential source for the development of novel agents against different types of breast cancer.

CHAPTER 1

INTRODUCTION

Globally, breast cancer remains the most diagnosed cancer and the main cause of cancer-related deaths among females (Bray et al., 2018), accounting for 23 % of total incidence and 14 % death rate. Approximately half of the breast cancer incidences and 60 % of the mortality rate are estimated to befall in developing countries (Jemal et al., 2011).

According to 2018 GLOBOCAN classification, female breast cancer tightly follows lung cancer with 11.6 % in both sexes. In contrast to other continents, the percentage of cancer deaths in Africa (7.3 %) and Asia (57.3 %) are higher than the incidence (5.8 % and 48.4 % respectively) because of the high casualty rates and the diverse distribution (Bray et al., 2018). A review on breast cancer care in Africa also showed an increase in breast cancer incidence mostly in low- and middle-income countries (Kantelhardt et al., 2015). The occurrence of breast cancer in Sub-Saharan Africa is quite low, but the survival rate is poor due to late diagnosis, lack of logistics, the expensive nature of the medications and treatments. Hence mortality rates are as high as in developed countries. Late stage in diagnosis has been found to be the main problem for breast cancer control. In Ghana, a study disclosed that 64.1 % of patients come with the late stage of the disease. The delay in diagnosis experienced in these countries is due to poor awareness, the high cost of available treatments, absence of organized early detection programs, poor facilities for accurate, timely diagnosis and treatment (Jedy-Agba et al., 2016).

Among all these factors, the treatment cost is the major reason for delay observed mostly in low-and middle- income countries. The affected individual often

falls on herbal products, and traditional medicine as one of their first treatment alternatives to alleviate the effect and/or as a preventive measure. Thus, access to operational and inexpensive cancer treatments in developing-countries would remarkably reduce mortality, particularly in locations where health-care amenities are less well established. However, the increasing cost of the cancer burdens are affecting the economies of even the richest nations, not to talk about the developing countries. This also places unbearable stresses on the health-care systems. Therefore, more commitment is desperately needed in preventive measures and to detect early cases for adequate treatments. In that regards, all governments were encouraged to show commitment in implementing high-quality screening and early detection programs that are according to him an investment rather than a cost (Stewart et al., 2016). He further emphasis the importance of these programs in low- and middle-income countries for protection of the populations. Stewart summarized the importance of such calls by saying that the battle against cancer will not be won with treatment only, but rather with an effective prevention measure.

Diet and nutrition are considered as effective strategy for the prevention of cancer. Epidemiological reports advised the consumption of fruits, soy products, and vegetables to reduce the risk of breast cancer (Li et al., 2017, Keating & Martel, 2018), and of some dietary natural products which might lessen the incidence and increase cancer survival rate (Nechuta et al., 2012; Farvid et al., 2016). Additionally, many of such products together with their bioactive compounds had been shown to inhibit cell proliferation, cause cell cycle arrest, induce metastasis, angiogenesis and apoptosis, to downregulate ER-α expression and finally sensitize breast tumors cells to chemotherapy and radiotherapy (Hu et al., 2014; Lv et al., 2014; Varinska et al., 2015; Gallardo & Calaf, 2016)

The bacterial anaerobic fermentation of plant dietary fiber in the large bowel produce short chain fatty acids such as butyrate which was seen to reduce cell proliferation and induce apoptosis in cell lines (Hague et al., 1995; Halestrap et al., 1997). Dietary fiber also might prevent colon cancer and interfere with the enterohepatic circulation of estrogens. Reduction was reported in androstenedione and circulating estrogen levels, and in mammary cancer growth, metastasis, and angiogenesis with high fiber intake (Raz et al., 2002).

In addition, fiber-rich food is important source of Phyto-estrogens and estrogen-like natural compounds which might interact with and control the activity of ERs, thereby limitating the risk of hormone-dependent tumors such as breast cancer (Adlercreutz et al., 1987; Monroe et al., 2007; Vieira et al., 2012).

Plant based polyphenols have numerous biological effects which include anti-inflammatory, scavenging of oxidative agents, antimicrobial activity, and inhibition of platelet aggregation (Wachowicz, 2005; Yoon & Baek, 2005; Zhang & Tsao, 2016). Together with phytochemicals, some minerals and vitamins such as carotenoids, folate, vitamins B6, C, D and E, and selenium, might reduce cancer risk by preventing oxidative damage and inhibit cell proliferation. These nutrients can further induce cell-cycle arrest, maintain DNA methylation, and/or modulate the concentrations of steroid hormone and hormonal metabolism (Vanaja et al., 2010; Williams et al., 2010; Beaver, & Dashwood, 2011; Song et al., 2015; Iqbal et al., 2017; Kapinova et al., 2018). Diallyl disulfide, and sulphoraphane can maintain DNA stability and enhance transcription by behaving as histone deacetylase inhibitors (Bianchini & Vainio, 2001; Ho et al., 2005). Bioactive compounds from plants such as resveratrol, curcumin, and isothiocyanates are known for their anti-inflammatory, antioxidant, and anti-carcinogenic activities (Chung et al., 2013; Hosseini & Ghorbani, 2015; Wang et al., 2015).

Tigernut "*Cyperus esculentus lativum*" is a tuber of family Cyperaceae which is underutilized. It produces rhizomes from the base of the tuber that is to some extent spherical (**Figure 1.1**). It grows freely and is consumed widely in Ghana, other parts of west Africa, parts of Europe particularly Spain and as well as in the Arabian Peninsula (Ejoh et al., 2006). Many thousand years ago, Tigernut was cultivated in region of chufa between Sudan and Egypt on the borders of the Nile River. Proof of Tigernut importance and value is shown archaeological searches that found earthen jars containing Tigernut in graves of pharaohs. Tigernut milk was classified as therapeutic drink due to its been highly energetic and diuretic, rich in mineral, vitamins C and E, and predominantly contain phosphorus and potassium (Ejoh et al., 2006).

Tigernut tubers appear somewhat long or round with a dimension of 8 mm to 16 mm (**Figure 1.1**). When hydrated, it is slightly harder more of a nut texture, but with a rather more intense and concentrated taste. It is cultivated twice a year in Ghana, but its cultivation time is between April and November. It needs continuous irrigation and must be properly dried before storage. The drying process is completely natural by sun drying and the process can take up to one month. The dehydrating process is to ensure longer shelf life, to prevent rot or bacterial infection. In all, it is to secure their quality and nutritional level. Unfortunately, the dehydration process makes the Tigernut skin wrinkled, a situation that limits its acceptability to some people (Belewu & Abodunrin, 2008). Three types of varieties of Tigernut are cultivated: black, brown and yellow (Umerie et al., 1997). The yellow variety is preferred over others because of its inherent properties such as attractive colour, large size, fleshier nature, low fat, and less anti-nutritional factors (Okafor et al., 2003). It also said to yield more milk upon extraction with protein content which is higher compared to the rest. It was

reported that Tigernut contain practically twice the amount of starch as potatoes tubers (Kuner et al., 2002).

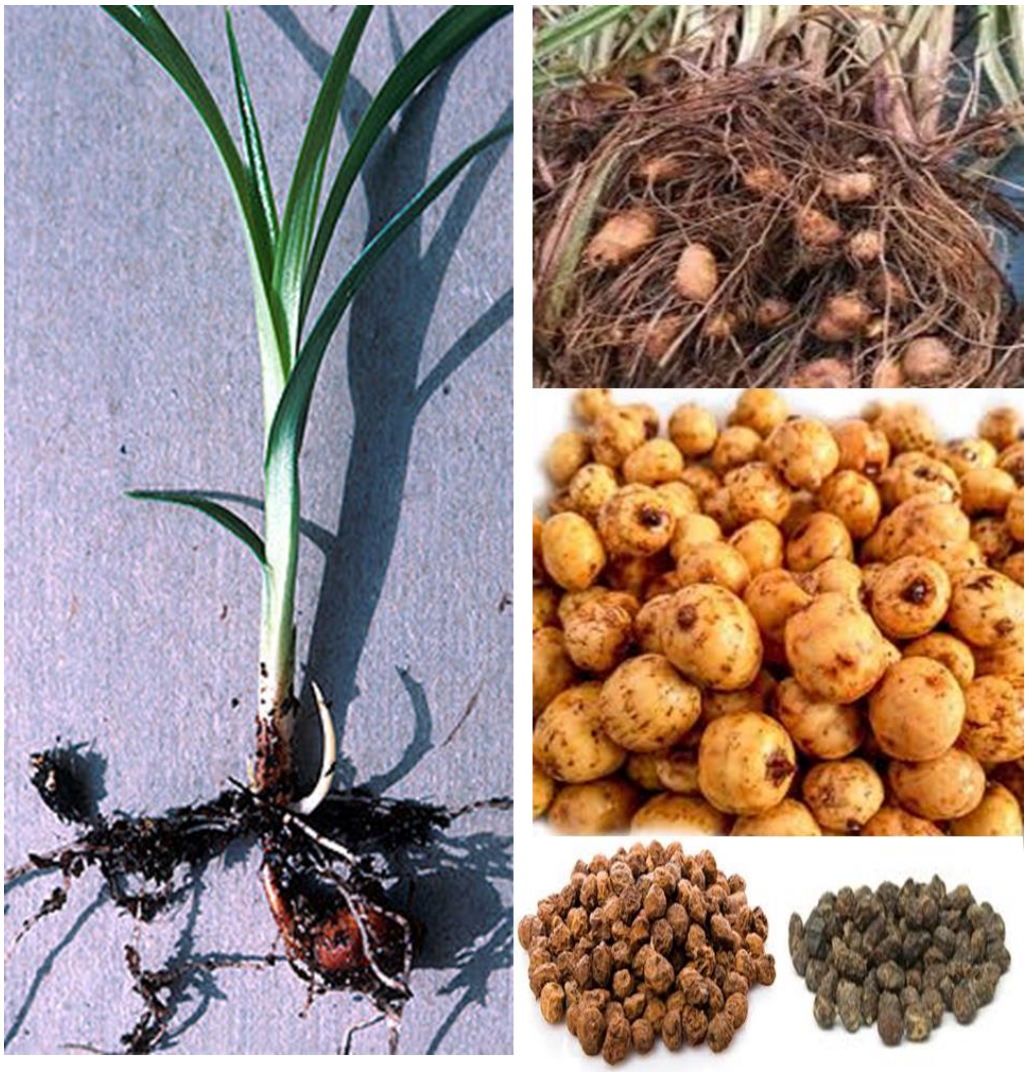

Figure 1. 1 Picture of Tigernut "*Cyperus esculentus lativum*" tuber and nuts

1.1. Research Rationale

Cyperus esculentus L., a tuber which is commonly used as snack and in the food industry in some part of the world like Africa, Spain, and America. It contains several vitamins, nutrients, fiber, mono-unsaturated fatty acids, and antioxidants that make it suitable to be part of an anti-cancer diet. It also contains compound such as ß-sitosterol, quercetin, fatty hydroxamic acid, oleic acid, and nutrients like vitamin D and E which are proven to have anti-inflammatory, antiproliferative, anti-metastatic, immune-stimulatory effects, and anti-cancer property. All these data increase the likelihood of C. *esculentus* to be a good candidate of an anticancer drug. Therefore, this work wants to determine the antiproliferative and migration activity of C. *esculentus*, on MDA-MB-231 and MCF7; and to elucidate the possible mechanisms.

1.2. Research objectives

The general objective of this work is to determine the antiproliferative and anti-migration activities of C. *esculentus* extracts, as well as to elucidate the mechanisms by which these activities could be achieved.

The specific objectives are as follows:

i. To determine the phytochemical groups that are present and the antioxidant activity of the aqueous and ethanolic extracts of Tigernut.
ii. To determine the effect of the extracts on the cell viability and the cell cycle progression of MDA-MB-231 and MCF7.
iii. To evaluate the effect of the extracts on the migration and invasion of MDA-MB-231 and MCF7.

iv. To elucidate the possible cell signaling mechanisms related to cell viability, cell cycle, cell migration and cell invasion at both mRNA and protein expression levels in both cell lines.

1.3. Flow chart of the research

The flow of the work is depicted in **Figure 1.2**. Two varieties of Tigernut were extracted in water and 70 % ethanol. The extracts were tested for phytochemicals and antioxidant activity. Also, MTT assay, cell cycle analysis, cell migration and invasion assays were performed. This is followed by the determination of genes and proteins expressions by qPCR and Western blotting.

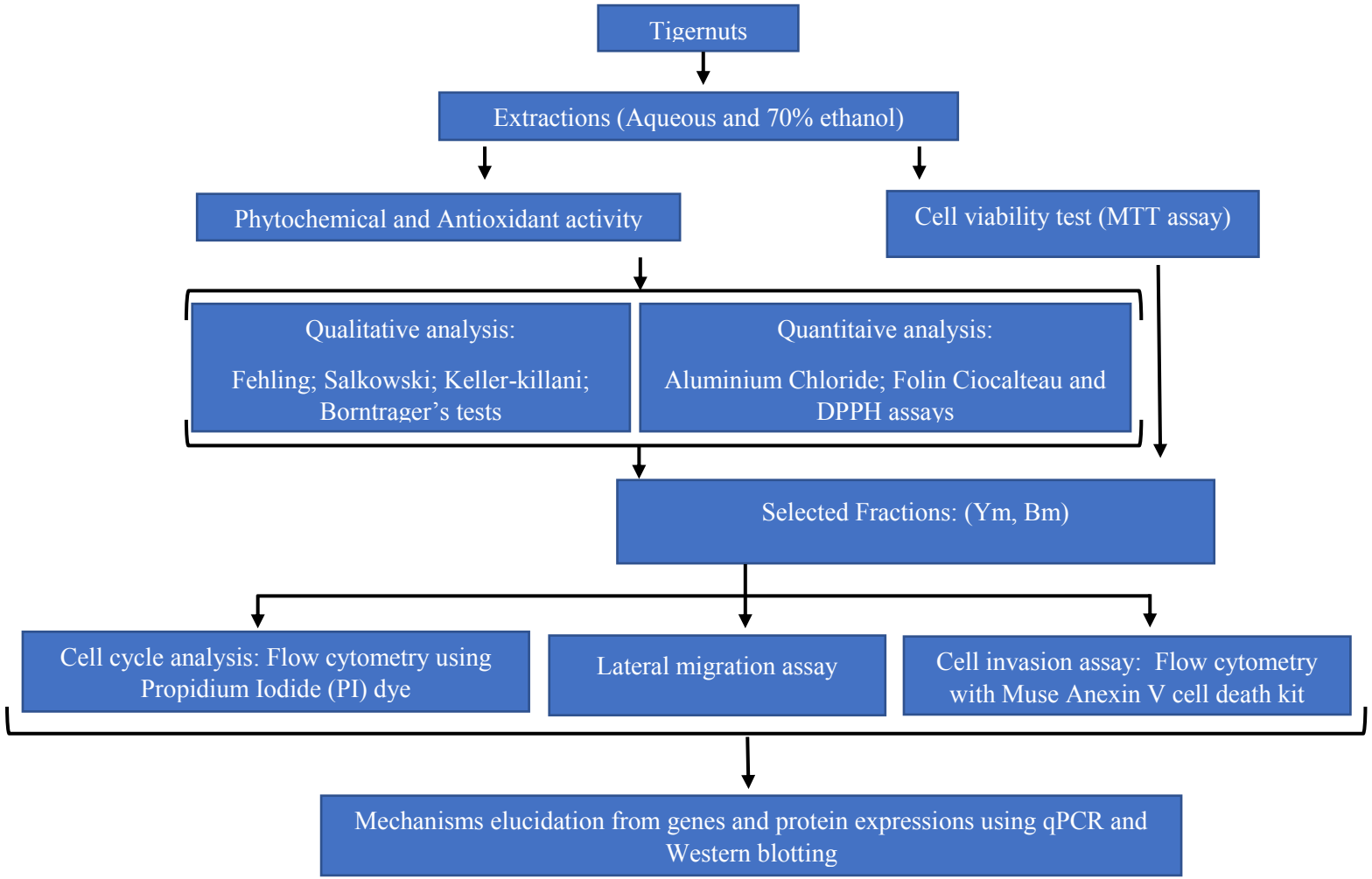

Figure 1. 2 Flow chart of Research

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

The cancer burden worldwide is expected to increase to 24 million in 2035 from 14 million cases observed in 2012 (Stewart et al., 2016). The higher percentage of this increment is projected to come from developing countries where cancer treatment and services are hard pressed. Due to the threat and the mortality rate increase, no country can find its way out of the cancer menace. Therefore, it is essential to include preventive and earlier detection measures in the cancer control improvement procedures. Around 40-50 % of cancers could have been prevented if an up-to-date knowledge about the risk factors was deciphered into the current public health policies (Stewart et al., 2016). Though progress is still needed in other areas such as weight control, physical activity, and mutation profiles, the progress in diet control with consumption of fruit, wholegrains, vegetables, and of less processed meat has helped in the reduction of mortality rate.

2.2. Tigernut

Tigernut (*Cyperus esculentus*) is a tuber known under numerous names such as: nut grass, earth or ground almond, yellow nut, and edible galingale (Sánchez-Zapata et al., 2012). It has an appetizing, sweet and nutty flavour and is consumed either fresh, semi-dried, and/or completely dried as snack. Tigernuts are mostly eaten raw in Ghana as snack, but it is a vital food crop within certain communities in some part of West Africa. To increase their uses, researches in the country have focused on their nutritional values either alone and/or as a composition of many diets' formulations (examples are their uses in baking, beverages and drinks production, cooking oil, and in cosmetic where the oil is mostly used for soap). Current

research and reviews on this plant have been focused mainly on the phytochemical compositions, organoleptic properties, biochemical activities, nutritional values, and mostly on the oil content and its characterizations. The medicinal values of Tigernut are rarely discussed, although it is widely claimed in folklore activities and is said to be a balance diet on its own.

Tigernut has excellent nutritional qualities with a fat composition like olives (Coşkuner et al., 2002) in addition of being cholesterol and gluten free. It is also said to be a promising ingredient for gluten-free baked product (Aguilar et al., 2014). Moreover, it is a rich plant source of flavonoids (El-Habashy et al., 1989) and rich in water, alkaloids, fibres, saponins, digestible carbohydrates, and fatty oils (glycerides) (Jeong et al., 2000; Ekeanyanwu et al., 2010). Additionally, Tigernut contain elements, like potassium, phosphorous, calcium, zinc, iron, manganese and magnesium (Addy & Eteshola, 1984; Coşkuner et al., 2002; Sánchez-Zapata et al., 2012). The fatty oil amount in Tigernut was 25-27 % and are classified into saturated fatty acids (myristic acid) and unsaturated fatty acids (linoleic acid (8.37 %), oleic acid (69.25 %), stearic acid (5.07 %) and palmitic acid (15.19 %)) (Eteshola & Oraedu, 1996; Salem et al., 2005; Adel et al., 2015). It is denoted that Tigernut had marked therapeutic effects for hyperplasia, intestinal metaplasia, and the animal experiments showed no toxic effect, so a safety guarantee was provided for its clinical application (Allouh et al., 2015; Stanley & Francis, 2018). Moreover, it is considered as analgesic, antimalarial, antibacterial, antimicrobial, astringent, carminative, antipyretic, diaphoretic, demulcent, diuretic, hypoglycemic, hypotensive, hepatoprotective, tonic, stomachic, vermifuge and as anti-inflammatory agent (Gupta et al., 1971; Atta & Zaman, 1989; Salem et al., 2005; Hanaa, 2007; Monago & Uwakwe, 2008; Nwaoguikpe, 2010; Gambo &, Dáu, 2014; Oyedepo et al., 2014). In *vivo* study of a food supplement with

Tigernut on mices which were induced with streptozotocin showed that Tigernuts had appreciable hypoglycemic and hypolipidemic effects (Hanaa, 2007). The comprehensive study on the lipid profiling of Tigernut oils by Adel et al. (2015) showed that Tigernut oils could substitute some common vegetable oils in the food industry.

2.3. Cancer Risk Factors

Cancer, in general, occurs because of individuals being exposed to carcinogenic agents in what they eat, drink, inhale and/or exposed to. Tobacco is known to be the most important risk factor for cancer which was reported to cause 22 % deaths globally and specifically 71 % for lung cancer (Stewart et al., 2014). Also, specific infections by Hepatitis B and C viruses, Human papillomaviruses, and Helicobacter pylori are other major risk factors.

For other major global cancers, reproductive behaviour and uses of hormonal drugs/products, along with differences in weight, diet, alcohol consumption and lack of exercise, are alleged to underlie the differences seen in the risk of breast cancer (Stewart et al., 2014). Increase of 10 % in the number of processed foods in the diet was related to a substantial rise of more than 10 % in risks of overall and breast cancer (Fiolet et al., 2018; Monge & Lajous, 2018). In Ghana, the prevalence of several of the behavioural risk factors is high and are increasing. Some studies showed that more than 85 % of Ghanaians consume less than the recommended five daily servings of fruits and vegetables (Bosu et al., 2015). Truly, vegetable and fruit intake in Ghana is among the lowest in the world (Hall et al., 2009).

2.4. Phytochemicals and Cancer prevention

Phytochemicals have shown potential in inhibiting experimental carcinogenesis in various type of cell lines. Most tested phytochemicals are from fruits, vegetables, herbs, and medicinal plants. These are phenolic compounds, terpenoids, flavonoids, carotenoids, and others (Thoppil & Bishayee, 2011; Iqbal et al., 2018). Often phytochemicals act as antioxidants especially the flavonoids, and some have anti-inflammatory, anti-hormone, immune-enhancing effects and anti-cancer property (Seeram et al., 2005; Iqbal et al., 2015; Iqbal et al., 2017). Further studies have shown their influence on cell cycle progress, cell differentiation, cell apoptosis, and on angiogenesis (Chung et al., 2013; Hosseini & Ghorbani, 2015; Wang et al., 2015).

2.4.1. Flavonoids

Flavonoids are a subgroup of polyphenols which can be found in fruits, coffee, tea, etc. Most of these are phytonutrients such as quercetin, kaempferol, and myricetin which are known to have some antioxidant properties with quercetin being the highest. All flavonoids are suitable chelating agents and tend to be potent antioxidants. Their role in cancer preventions has been mostly supported by epidemiological studies and few animal studies.

Dietary flavonoids are these days mostly sought after because of their cost-effectiveness, safety, and feasibility of oral administration. According to Kuo, the biological activities of dietary flavonoids depend on their structure and possible synergistic action with chemotherapy agents (Kuo, 1997). Further examples were also given on some flavonoids (Isoflavones) that have a weak affinity for the estrogen receptor while others do bind to such receptors (e.g. low-affinity type II estrogen binding sites), but the mechanism is unknown. Fisetin, a nutritional flavonoid found

in many vegetables and fruits is shown to prevent cancer development by affecting their cell cycle, inhibiting their invasion and metastasis, inducing apoptosis and angiogenesis; all these with no toxicity effect on the normal cells (Lall et al., 2016). It has been reported that cell division of MDA-MB-231 was inhibited by fisetin and apoptosis was conducted through mitochondrial membrane depolarization and the activation of CASP9, CASP8, and the cleavage of PARP 1 (Smith et al., 2016). Likewise, quercetin, another flavonoid compound, has also been shown to cause the similar cleavage and caspases responses in both MCF7 and MDA-MB-231 breast cancer cell lines through the expression of Bax and cyt C release (Chien et al., 2009; Ranganathan et al., 2015). Equally, genistein is seen to cause the inhibition of MDA-MB-231 cell growth via Bax and p21WAF1 molecular pathways (Li et al., 1999; Sarkar & Li, 2003). Genistein is also involved in the G2/M cell cycle and so many other pathways (Pan et al., 2012). Studies have been conducted paving the way to up-to-date information on effects of different flavonoids against various cancer cell types and their mechanism(s) of action elucidated (Gupta et al., 2015; Maggioni et al., 2015; Venturelli et al., 2016; Russo et al., 2017).

2.4.2. Sterols

Phytosterols or plant sterols are well known to improve blood lipid profile thus protecting against cardiovascular disease (Gylling et al., 2014). These days, insights are being gained to their role in cancer prevention and treatment. Phytosterols are shown to reduce cancers of breast, lung, stomach, liver, prostate, and ovary. While their exact mechanisms of action are not well delineated, phytosterols have been proposed to cause inhibitory effects through cell cycle arrest, invasion, and metastasis (Ramprasath & Awad, 2015). When consumed, phytosterol competes with the body's cholesterol for absorption because of the similarity in structure. By blocking

cholesterol absorption, phytosterols reduce the blood cholesterol level. During human disease processes such as obesity and cancer, a lot of rewiring is done to alter membrane composition. A review showed the importance of phytosterol against cancer initiation and growth (Woyengo et al., 2009).

Among these phytosterols are beta-sitosterol, stigmasterol, campesterol, and cycloartenol of which beta-sitosterol is the most abundant. β-sitosterol caused G2/M arrest phase and induced apoptosis via ERK-independent and phosphatidylinositol 3-kinase/Akt pathways (Moon et al., 2008). Same apoptotic activity was observed in MCF7 and MDA-MB231 cell lines (Awad et al., 2007). Their effect on the plasma membrane and lipid metabolism in relation to their protective activity against cancer has also been reviewed by (Zhuang et al., 2005; Hąc-Wydro et al., 2007; Cheng et al., 2018; Fakih et al., 2018). Lupeol and stigmasterol have also been shown to possess anti-cancer activity in addition to their already known anti-inflammatory activities. The in-*vivo* study has shown that these compounds inhibit cell viability, and migration, and cell morphogenesis via tumour necrosis factor-α (TNF-α), and VEGFR-2 signaling pathways (Kangsamaksin et al., 2017).

2.4.3. Steroids

Steroids are compounds made naturally or artificially that are used as medications for different types of illnesses (Man et al., 2010; Yue et al., 2013; Iqbal et al., 2015). Steroids play an important part in the management of stress, by responding to reacting inflammation, infection, and a host of other functions which ranges from blood pressure to blood sugar controls (McEwen & Kalia, 2010; Liu et al., 2017). When used together with chemotherapy drugs for instance in NHL treatment, steroids help prevent the side effects of the treatment, to stop allergic reactions and increase

appetite. Most often, corticosteroids are the type of steroids used in cancer treatment. Corticosteroids are compounds naturally produced by the adrenal glands. It helps control chemotherapy-induced vomiting and nausea, treat shortness of breath in advanced cancer, treat superior vena cava obstruction, and reduce swelling in case of cancer metastasis to the brain (McEwen & Kalia, 2010). Steroids are referred to as the unsung heroes of cancer care because it works quietly behind the scene to minimize, and prevent complications, and even helping in the success of other therapies while radiation therapy, chemotherapy, immunotherapy, and targeted therapies get all the praise for killing cancer.

2.4.4. Terpenoids

As a major group of secondary metabolites, triterpenoids are shown to possess important pharmacological activities such as antioxidant, hypolipidemic, anti-inflammatory, antibacterial, gastroprotective, immunoregulatory, antiviral and anticancer effect. In addition, terpenoids are said to be involved in the tumour cell survival, and proliferation, invasion and metastasis, and radio-resistance. Also, a review of the biological effects of terpenoids highlighted their chemo-preventive effect on breast epithelial carcinogenesis (Rabi & Bishayee, 2009).

A diversity of terpenoids has been shown to possess pharmacological activity against cancer. Examples are taxanes obtained from *Taxus brevifolia*, vincristine and vinblastine from *Catharanthus roseus* (Cragg & Newman, 2005; Srivastava et al., 2005). Alimentary monoterpenes likewise showed anti-tumour activity in preventing the development or progression of cancer, and in degenerating existed malignant tumours (Bardon et al., 1998). Moreover, terpenoids have been shown to exert cytotoxicity effect on liver cancerous cells in *in-vitro* and studies *in-vivo*, in which

their cellular and molecular mechanisms involved have also been elucidated (Thoppil & Bishayee, 2011). Certain reviews have dealt with the status and prospects of the relation between terpenoids and cancer prevention (Huang et al., 2012; Patlolla & Rao, 2012). Oleanic acid, a naturally occurring pentacyclic triterpenoid with anti-angiogenic activity (Sogno et al., 2009), re-establishes the homeostatic control of cell proliferation together with rosemary terpenoids and both reduce cancer risk through distinct mechanisms (Telang, 2018).

2.4.5. Tannins

Tannins are a group of astringent, and polyphenolic biomolecules that bind to and react with proteins and several other organic compounds such as alkaloids and amino acids. Due to their binding properties to proteins, pigments, basic compounds, metallic ions, large-molecular compounds and their display of anti-oxidant activities, tannins have been subject of research for their anti-cancer properties (Barrajón-Catalán et al., 2010; Widsten et al., 2014; Abu et al., 2016). Tannins have been found to affect the endoplasmic reticulum (ER) stress, to cause apoptosis in prostate cancer cells (Nagesh et al., 2018), HER^+ breast cell line (Jordan & Booth, 2018), ER^+ (Booth et al., 2013; Ngobili et al., 2015) and fatty acid synthase over-expressed human breast cancer cells (Nie et al., 2016), and to induce G1/S phase arrest and inhibit different pathways (Darvin et al., 2015; Darvin et al., 2017).

2.4.6. Saponins

Mostly plant-based phytochemical, saponins can also be found from marine organisms. Due to their structural compositions, it has been seen to exhibit pharmacological effects among which are antioxidant and anticancer activities (Bang et al., 2005; Beit-Yannai et al., 2011; Vuong et al., 2015). Saponins were found to

prevent the proliferation of cancerous cells by interfering with the replication of cellular DNA and causing cell cycle arrest (Liu et al., 2014). It also induces CASP3 cleavage, thus apoptosis activation (Beit-Yannai et al., 2011). Furthermore, saponins were shown to possess a cytotoxic effect on multidrug resistance (MDR) and non-MDR cells (Park et al., 2010). A review by Isil et al. (2015) has highlighted the anticancer activity of various plants issued from their rich content of tannin and saponin. Similar reporting on the chemical structural and medical applications of saponins showed ginsenosides, and dioscin to be strong anti-tumour agents in inhibiting tumour angiogenesis, invasion, and metastasis, and causing cell cycle arrest (Shuli et al., 2010; Ong et al., 2015).

2.5. Breast cancer

Globally an increase in breast cancer incidence rate has been observed. For instance, in 2012, approximately 1.7 million cases were detected which represented 12 % of total cancer cases and 25 % of cancer cases in women (Ferlay et al., 2015; Kantelhardt et al., 2015). This is a rise from 2010 when 1.6 million new cases of BC were detected (Forouzanfar et al., 2011), and accounted for over 500,000 deaths annually.

A 2011 study from the American Cancer Society stated breast cancer as the most detected cancer in women, representing a change from earlier years with cervical cancer been the most diagnosed cancer in Sub-Sahara countries (Jemal et al., 2011). This analysis is consistent with cancer data reported in Cote d'Ivoire (Echimane et al., 2000), Uganda (Gakwaya et al., 2008; Wabinga et al., 2013; Galukande et al., 2014), Nigeria (Akarolo-Anthony et al., 2010; Stefan et al., 2013), and Ghana (Stefan et al., 2013; Thomas et al., 2017; Vanderpuye et al., 2017a). Though the occurrence rate

observed in the developing countries is low compared to the developed countries, the survival rate is poor resulting in a high mortality rate of about 75 % (Vanderpuye et al., 2017b). Late report to the hospital with aggressive tumours, cost of treatment, lack of therapeutic options and lack of information are some of the causes of such high rate.

2.5.1. Breast cancer subtypes

Breast cancer is usually categorized into estrogen receptor positive (ER^+) and estrogen receptor negative (ER^-). The use of biomarkers, for instance, human epidermal growth factor receptor 2 (HER2), and progesterone receptor (PR) further divided breast cancer into several molecular subtypes, for example, luminal A and B, HER2-positive and basal-like breast cancers (**Table 2.1**) (Holliday & Speirs, 2011). The latter is considered as triple-negative breast cancer (TNBC) in some cases because it lacks the expression of ER, HER2, and PR. This makes it non-responsive to most available treatments hence making it extremely intractable.

Earlier studies found that Ghanaians have a threefold rise in TNBC incidence in comparison to African Americans, and White Americans. Explicitly, 82 % of Ghanaians were detected with TNBC against 26 % African Americans and 16 % White Americans (Stark et al., 2010; Seshie et al., 2015). Likewise, 49.4 % of tumours tested at Korle-Bu teaching hospital were found to be hormone-receptor-negative, with triple negative being the subtype most detected (Ohene-Yeboah & Adjei, 2012; Seshie et al., 2015). The same consistency is reported from a study in Kumasi metropolis between July 2004 and June 2009 where 42.5 % triple negative tumour was spotted from 54 breast cancer cases (Stark et al., 2010; Ohene-Yeboah & Adjei, 2012).

Table 2.1 Molecular classification of breast cancer (Holliday & Speirs, 2011)

Subtype	Immuno-profile	Characteristics	Example cell lines
Luminal A	ER+, PR+/-, HER2-	-Low expression of proliferation marker Ki67 -Responsive to hormone therapy -Often responsive to chemotherapy	MCF7, T47D, SUM185
Luminal B	ER+, PR+/-, HER2+	-High expression of proliferation marker Ki67 -Usually responsive to hormone therapy -Variably responsive to chemotherapy -Variably responsive to HER2 antibody therapies (i.e. trastuzumab)	BT474, ZR-75
Basal	ER-, PR-, HER2-	-High expression of proliferation marker Ki67 -Expression of EGFR+ -Variable expression of a basal cell marker cytokeratin 5/6 -Not responsive to hormone therapy, but often responsive to chemotherapy	MDA-MB-468, SUM190
Claudin-low	ER^-, PR^-, $HER2^-$	-Ki67, E-cadherin, claudin-3, claudin-4 and claudin-7 low. -Intermediate response to chemotherapy	BT549, MDA-MB-231, Hs578T, SUM1315
HER2 amplified	ER-, PR-, HER2+	-High expression of proliferation marker Ki67 -Often responsive to HER2 antibody therapies (i.e. trastuzumab) and chemotherapy	SKBR3, MDA-MB-453

EGFR, epidermal growth factor receptor; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor.

2.4.1(a) MDA-MB-231 cell line

MDA-MB-231 are cancerous breast cell lines that are being used as models for numerous experimental studies (Jiang et al., 2016). Since these cells have a variety of mutated genes (e.g. p53, BRCA, CDKN2A, BRAF, and RAS), and express EGFR and TGFα, MDA-MB-231 are commonly used for testing the effects of possible anti-cancer agents on the affected pathways (Huovinen et al., 2011; Jiang et al., 2016). It is an epithelial cell line derived from a pleural effusion of a Caucasian female with metastatic mammary adenocarcinoma. MDA-MB-231 is TNBC making it highly aggressive and invasive. Classified first under the basal breast cell line, it is now referred to as claudin-low molecular subtype breast cancer line because of the downregulation of claudin-4 and claudin-3 proteins and the low expression of Ki-67.

2.4.1(b) MCF7 cell line

MCF7 is a cancerous breast cell line that is not as aggressive as MDA-MB-231. It was isolated from a 69-year old Caucasian woman in 1970. The cells do retain quite a few features of differentiated mammary epithelium and can process estradiol through cytoplasmic ERs. Their growth is said to be inhibited by TNFα. MCF7 cells are estrogen receptor positive breast-origin cells. Omega-3 and omega-6 fatty acids such as docosahexaenoic acid (DHA), arachidonic acid (AA), and eicosapentaenoic acid (EPA) have shown to inhibit MCF7 cell growth, and proliferation (Mansara et al., 2015). MCF7 cells have a mutation in PIK3CA and CDKN2A genes which make them be a tool in research concerning the signaling pathways in which these factors are involved.

2.5.2. Breast cancer, risk factors, and diet

The onset and development of breast cancer could be affected by many exogenous and endogenous factors. Exogenous factors comprise reproductive, lifestyle and environmental factors such as smoking, excessive intake of red meat and alcohol, overweight, lack of exercise, etc. Genetic risk factors, for example, mutations on breast cancer susceptibility gene 1 and 2 (BRCA1 and BRCA2), account for only about 5–10 % of all breast cancer occurrences.

A long period of exposure to estrogens is also associated with a bigger risk of breast tumour. Though the mechanisms are not well grasped, the accepted theory proposed that estradiol (E2) acts through ERα in inducing cell proliferation and initiating mutations from replicative mistakes taken place in pre-mitotic DNA synthesis. These cause an increase of mutated cells which, over a period accumulate to encourage neoplastic transformation. The second theory is based on the genotoxic effect of estrogen metabolite, a non-receptor mediated mechanism. According to a review by Yue et al. (2013), both mechanisms are supported by the weight of evidence available. In addition, it was said that estrogen metabolites probably control the function of stem cell and cancer growth. The group concluded that the effect of ER-dependent and -independent in cancer development need to be considered in the choice of treatment because anti-estrogens only block receptor-mediated pathways while aromatase inhibitors can block both receptors- and non-receptor-mediated mechanism (Yue et al., 2013). As it is, the percentage of risk from both the gene mutations and estrogens effect is then found to be low compared to the exogenous factors. Therefore, prevention of breast cancer ought to target mostly the exogenous factors especially lifestyle and diet.

Diet and lifestyle are indeed important risk factors that can be prevented and must be considered in all preventive measures. In this regard, efforts are being made by various authorities/organizations, including the Ghana Ministry of Health, to promote a good lifestyle and healthy diet. Similarly, research conducted to explore new anti-cancer treatments are now focusing on natural compounds from the regular human diet since these compounds do not often exhibit severe side-effects due to their synergic effect when mixed with the other compositions of the diet; but rather act efficiently on a varied range of molecular targets involved in carcinogenesis.

Prospective studied have revealed that eating patterns characterized by increased intakes of fruits, whole grain foods, and vegetables, and lower consumptions of processed, red meats and salt, are linked to reduced dangers of cancer, and death. Thus, a healthy diet might improve the overall survival rate after the diagnosis of breast cancers. Taking all these into account it was ratified that individuals should have a healthy diet to lessen the risk of cancer (Fung et al., 2013). They ought to eat a lot of whole grains, fruits, legumes, and vegetables; limit foods high in salt and red meat; processed meat and avoid sugary drinks; and limit high-calorie foods (Norat et al., 2015; Schüz et al., 2015). Other case studies also connect a healthy diet to the reduction in breast cancer risk predominantly in postmenopausal women (Catsburg et al., 2015) and of ER-negative/PR-negative and ER-negative/PR-positive tumours (Dong et al., 2011). A collective analysis of carotenoid level in the circulating blood further showed a lower risk of breast cancer mainly on ER-negative tumours (Eliassen et al., 2012). Liu and his/her team correspondingly found an inverse relationship between the intake of vegetable protein and fat, dietary fibre, and nuts consumed during adolescence with adult breast cancer risk (Liu et al., 2014).

2.5.3. Breast cancer and available treatments

Treatment for breast cancers is administered according to the type, the stage and how far it has been spread. Chemotherapies are often used in the early stage but can be added to radiotherapy sections when the stage is advanced. Hormone therapies like anti-estrogen therapy and targeted therapies (such as the use of Herceptin, Avastin) are also among the medications for breast cancer treatment. Surgical resection such as lumpectomy and mastectomy are performed most on late stages of breast cancer. People with breast cancer often get more than one kind of treatment. The treatments come either as the main course or as a combination depending on the type and stage of cancer and the patient condition. Notwithstanding, the occurrence of drug resistance and strong side effects has hampered the efficiency of these treatments. Also, the cost of the available treatment makes it difficult for the average people to access it.

2.6. EGFR signaling pathways

Many specific molecular targets have been investigated to design new therapies that will affect tumour growth and survival. Among such is EGFRs which are transmembrane receptors. These receptors are present on the cell membranes and have been found to regulate a lot of signal transduction cascades when bound to epidermal growth factors (EGF) (**Figure 2.1**).

The EGFR family consisted of EGFR1, HER2, EGFR3 and EGFR4 have shown different rates of overexpression (EGFR1 in 16.4 %, EGFR3 in 17.5 %, EGFR4 in 11.9 % and HER2 in 22.8 %,) (Wilson et al., 2009). EGFR1, HER2, and EGFR3 expression levels were inversely related to that of estrogen receptor (ER) (Witton et al., 2003). Upon activation by their specific ligands, EGFRs stimulate intracellular signalings, for example, the Ras/Raf/MAPK (**Figure 2.2**), JAK/STAT, PI3K-AKT and

PLCγ. At the cellular level, EGFR induces cell proliferation, protect against apoptosis and alter the cell adhesion and motility; while at the physiological level, it encourages invasion and angiogenesis (Masuda et al., 2012).

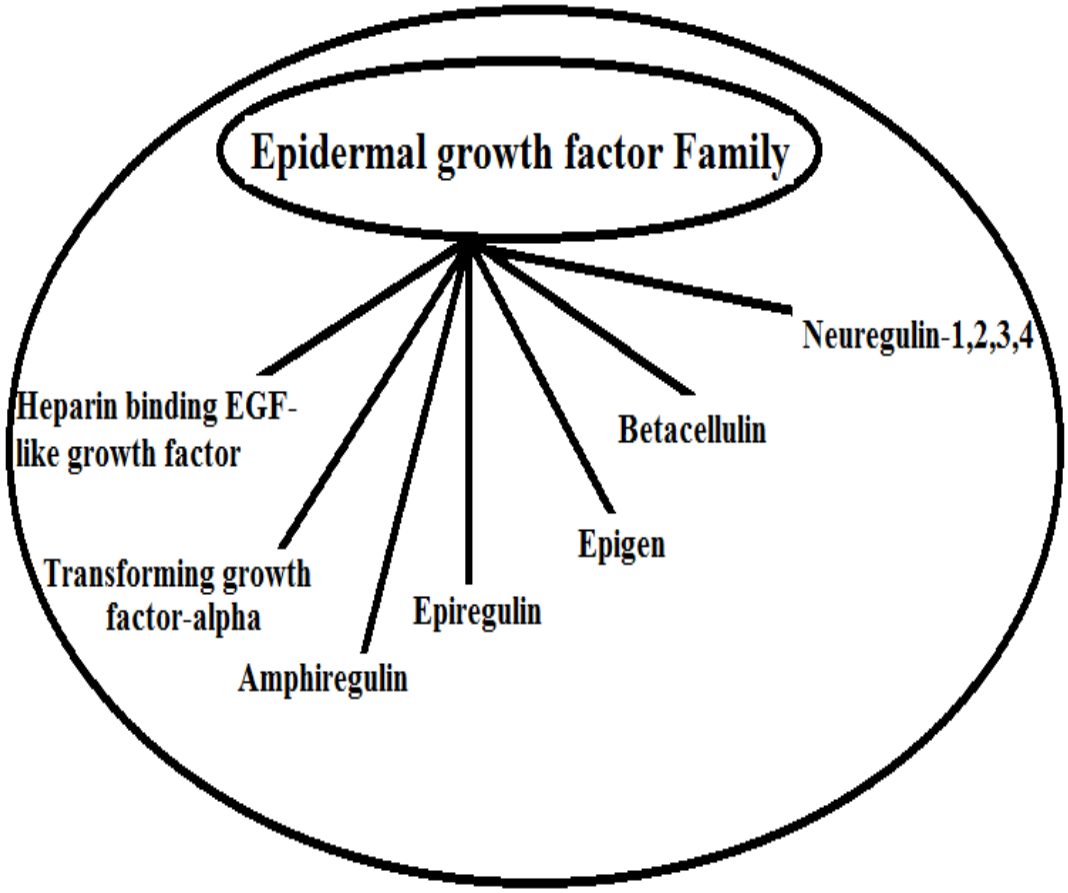

Figure 2. 1 The family of the epidermal growth factor (EGF) (Gupta & Chaphalkar, 2014)