

EFFECT OF QUERCETIN LOADED NANOPOROUS SILVER HYDROXYAPATITE AS SUITABLE NANOCARRIER FOR THE ANTI PROLIFERATION OF CANCER CELLS

Submitted in partial fulfillment of the requirements
for the award of

**Master of science
In
Medical biotechnology and
Clinical research**

by

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RESEARCH

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the Bonafide work of **Ms. KESAVARTHNI G** who carried out the project entitled "**EFFECT OF QUERCETIN LOADED NANOPOROUS SILVER HYDROXYAPATITE NANOPARTICLE AS SUITABLE NANOCARRIER FOR THE ANTI PROLIFERATION OF CANCER CELLS**" under my supervision from 2022-2023

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DECLARATION

I, **Ms. KESAVARTHNI G (41101007)** hereby declare that the Project Report entitled "**EFFECT OF QUERCETIN LOADED NANOPOROUS SILVER HYDROXYAPATITE NANOPARTICLES AS SUITABLE NANOCARRIER FOR THE ANTI PROLIFERATION OF CANCER CELLS**" done by me under the guidance of **Dr. A. Madan Kumar**, Assistant Professor (Research), Center for Molecular and Nanomedical Sciences is submitted in partial fulfillment of the requirements for the award of Master of science biotechnology and clinical research.

DATE: 05.05.2023

PLACE: Chennai



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ABSTRACT

Cancer is still the second leading cause of death on Earth, with a large number of attempted treatments. Nanomaterials-based cancer therapy plays a significant role in increasing the therapeutic efficiency of anticancer drugs, reducing the side effects and targeted delivery of the drug payloads. The present study was aimed to study the anticancer effect of the Quercetin by formulating with Silver Hydroxyapatite nanoparticles for the effective treatment against the cancer cells. Silver Hydroxyapatite nanoparticles were synthesized by chemical precipitation method and the size of the nanoparticle was characterized by Scanning electron microscope, X-Ray Diffraction, Fourier-transform infrared spectroscopy and UV-spectroscopy. Furthermore, Silver hydroxyapatite nanoparticles were conjugated with Quercetin. In addition, the cytotoxic effect were seen in different cell lines and embryotoxic, teratogenic effects of conjugated drug on the development of zebrafish embryo were investigated in this study. It was observed that conjugated silver hydroxyapatite with Quercetin showed enhanced cytotoxicity against cancer cell lines without any toxic effect on normal kidney cells. Conjugated silver hydroxyapatite with Quercetin showed significant higher apoptosis of cancer cells,. The obtained results suggests that the conjugate drug initiated cytotoxicity through apoptotic death in cancer cells. The embryotoxic studies in zebrafish embryos revealed the optimum concentration as 1-2mM. These findings suggest that conjugated silver hydroxyapatite with Quercetin affected the development of zebrafish embryos only at relatively higher concentrations. The experimental results demonstrate that Conjugated silver hydroxyapatite with Quercetin has the potential to improve anticancer efficacy against cancer cells by inducing apoptosis in cancer cells without any effect on normal kidney cells.

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LIST OF ABBREVIATIONS

BC – Breast Cancer.

GLOBOCAN – Global Cancer Incidence, Mortality and Prevalence.

CRC – Colorectal Cancer

NP – Nanoparticle

PVA – Polyvinyl Acetate

HAp – Hydroxyapatite

Qu – Quercetin

AgHAp – Silver Hydroxyapatite

AgHAp-Qu - Silver hydroxyapatite conjugated with Quercetin

DPPH - α , α -diphenyl- β -picrylhydrazyl

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INTRODUCTION

1.1 CANCER

An enigmatic and dreadful disease or group of diseases is cancer. Multicellular organisms have been plagued by cancer for more than 200 million years, and evidence of cancer in the progenitors of contemporary human's dates back more than a million years (Hausman, D. M. (2019). Cancers develop when somatic cells accumulate mutations in the genes that control and limit cell division, survival, repair, motility, and invasion. The process is "Darwinian" in which oncogenic mutations happen randomly and the fate of the mutant cells and their offspring is determined by their relative "fitness," in this case, their capacity to survive, proliferate, and eventually disseminate from their required somatic niche). Worldwide, and in nations of all financial levels, cancer is the leading cause of death. As populations expand, get older, and embrace riskier lifestyle choices, the number of cancer diagnoses and deaths is predicted to climb dramatically, adding to the burden already present. This is crucial in low- and middle-income countries (LMIC), which are going through an economic transition that involves growing mechanisation of work and transportation, cultural changes in how women are viewed, and increased exposure and access to global markets (Torre et al., 2016). One of the most feared diseases of the 20th century, cancer is still spreading and becoming more common in the 21st.

1.1.1 INCIDENCE

The population-based cancer registries (PBCRs) and hospital-based cancer registries (HBCRs) of the National Centre for Disease Informatics and Research (NCDIR) of the Indian Council of Medical Research (ICMR; ICMR-NCDIR-NCRP), Bengaluru, have been performing the systematic collection of data on cancer in India since 1982 (Appendix). NCRP started out with the goal of gathering trustworthy data on the prevalence and trends of cancer. Numerous NCRP cancer reports from various registries in India have been released. 14,61,427 incident cases of cancer were found to be the projected number in India for 2022 (the crude rate was 100.4

per 100,000). In India, one in nine individuals has a lifetime risk of developing cancer. The most common cancers in men and women, respectively, were lung and breast cancers. Lymphoma was the most common site among children (0-14 years) with cancer (boys: 29.2%; girls: 24.2%). According to estimates, there will be 12.8% more cancer cases in 2025 than there were in 2020. Fig 1.1 depicts the Estimated proportion of top 10 leading sites of cancer in India by sex – 2022.

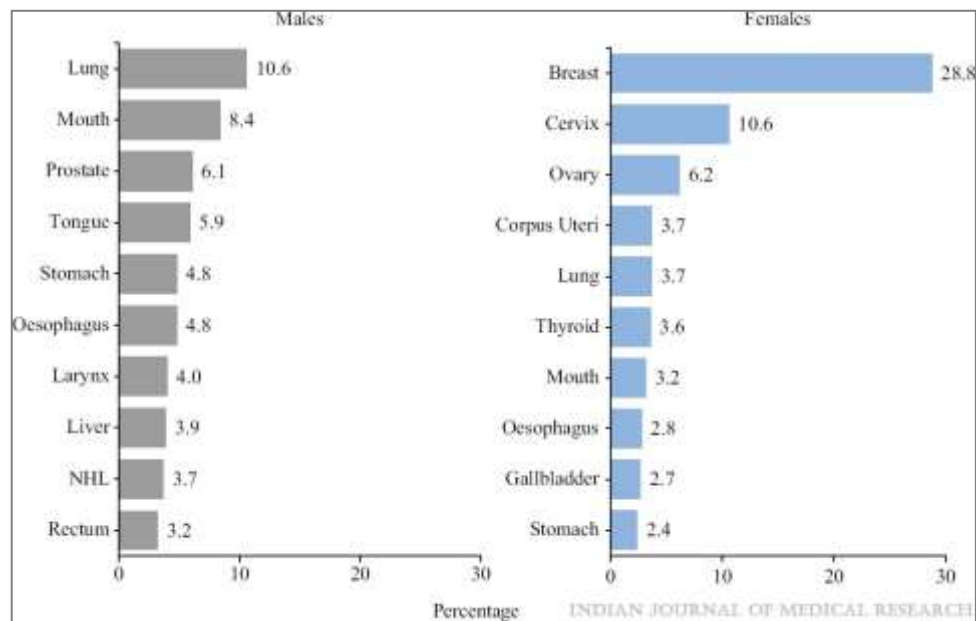


Figure 1.1- Estimated proportion of top 10 leading sites of cancer in India by sex – 2022 (Sathishkumar et al., 2022).

Fig 1.2 presents the estimated top five cancer-causing organs in men. These were the tongue (5.9%), stomach (4.8%), prostate (6.1%), mouth (8.4%), and lung (10.6%). Breast cancer accounted for 28.8% of all female cancers, followed by cervix (10.6%), ovary (6.2%), corpus uteri (3.7%), and lung (3.7%). While thyroid (3.6%) and gallbladder (2.7%) cancers were in the top ten among females but not males, liver cancer (3.9%) was in the top ten malignancies in men but not in women.

Males				Females			
Cancer site (ICD 10)	2015	2020	2025	Cancer site (ICD 10)	2015	2020	2025
Lung (C33-34)	63,087	71,788	81,219	Breast (C50)	180,252	205,424	232,832
Mouth (C03-C06)	50,779	57,380	64,519	Cervix (C53)	65,978	75,209	85,241
Prostate (C61)	36,419	41,532	47,068	Ovary (C56)	38,607	43,886	49,644
Tongue (C01-C02)	35,336	39,902	44,861	Corpus uteri (C54)	23,175	26,514	30,121
Stomach (C16)	28,815	32,713	36,938	Lung (C33-34)	23,163	26,490	30,109
Others	387,301	436,106	488,970	Others	296,027	335,235	378,271
All sites	601,737	679,421	763,575	All sites	627,202	712,758	806,218

ICD, International Classification of Diseases 10th Revision

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Figure 1.2 -Estimated trends in number of cancer cases for the leading sites (ICD-10 codes) in India in 2015, 2020 and 2025. (Sathishkumar et al., 2022).

Fig 1.2 gives the expected main sites of cancer cases over three different timeframes (2015, 2020, and 2025). While all site cancers in men are predicted to rise to 7,63,575 in 2025 from 6,01,737 in 2015, they are predicted to rise to 8,06,218 in 2025 from 6,27,202 in 2015. For the year 2025, it is anticipated that there will be 81,219 and 2,32,832 instances of lung and breast cancer in men and women, respectively. On average, cancer cases across all sites are predicted to rise by 27.7% between 2015 and 2025 (Sathishkumar et al., 2022).

It has only lately come to light that cancer can develop from small cell populations that are distinct from other progenies, despite the fact that the theory was first put forth decades ago. These populations not only possess the capacity for self-renewal but also the capacity for differentiation, which results in the emergence of a variety of phenotypes. Heterogeneous tumours are formed as a result, as seen in clinical settings. These tiny cell populations have been referred to as cancer stem cells (CSCs) due to their resemblance to somatic stem cells. Following its demonstration in leukaemia, CSCs have since been linked to other solid tumours, including gastrointestinal, brain, and breast malignancies (Miyoshi et al., 2010).

According to the idea of cancer stem cells, heterogeneous cells with the capacity for self-renewal, differentiation, and homeostatic regulation develop into tumours. The infinite capacity to self-renew or give rise to progenitor and differentiated cells characterises normal stem cells, which are tissue-specific cells. The development, growth, and reproduction of animals depend heavily on the proper management of these traits. Therefore, stem cell-like cells or the stem cell's precursors, which

typically only go through a few rounds of cell division after receiving genetic and epigenetic changes, could be the source of cancer (Garofalo and Croce, 2015).

The initial cells with the mutated transforming potential in the majority of malignancies are unknown. However, there are many indicators that point to the possibility that cancer could develop from (a) cancer stem cells; (b) bacteria acquiring and hybridising host DNAs; (c) embryonic rest; (d) maturation arrest; (e) dedifferentiation of mature cells; (f) stem cell mutations; (g) transformation of progenitor cells. The importance of gene alterations in the development of cancer has long been acknowledged (Yin et al., 2021).

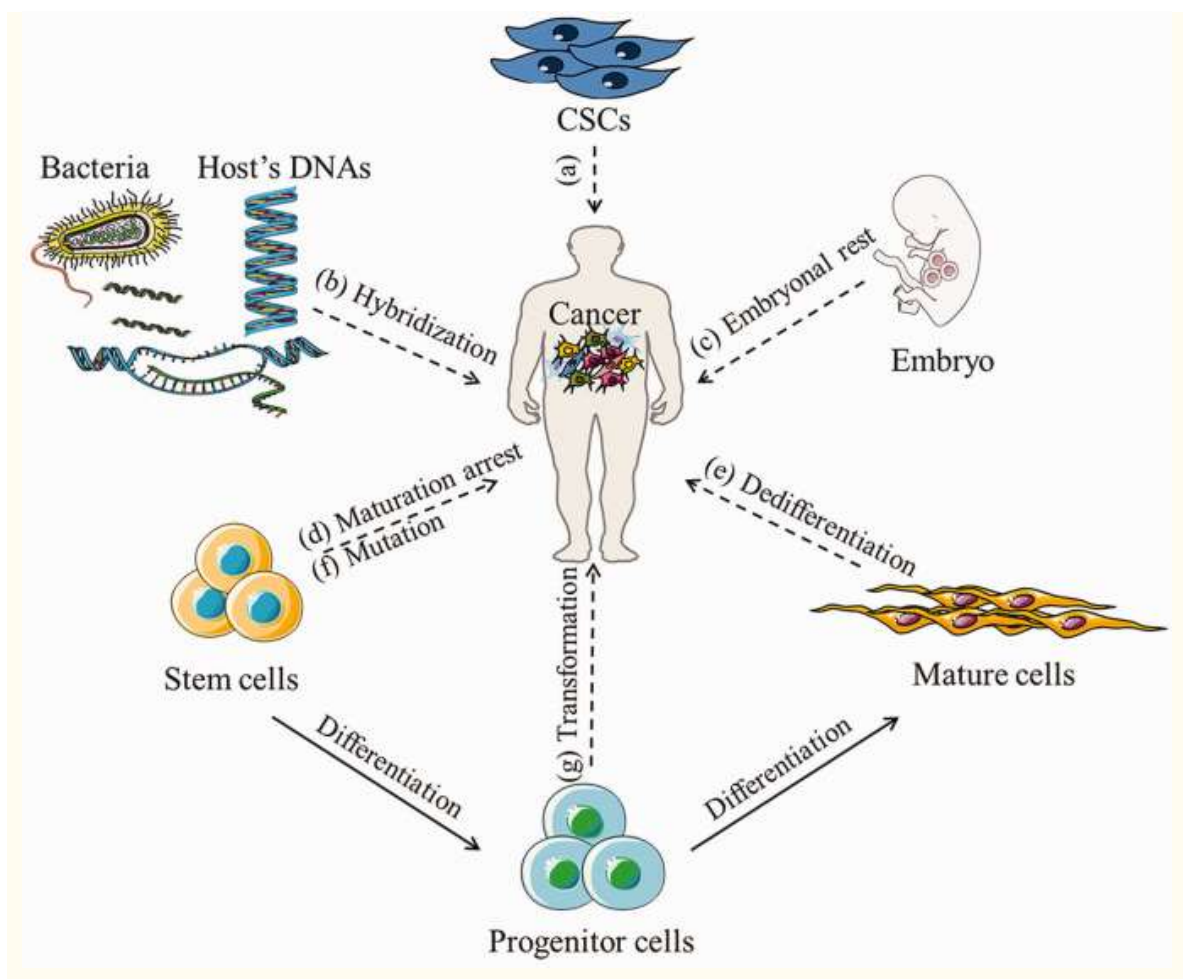


Figure 1.3 Possible cell origins of cancer. (a) In a clonal evolution concept, stepwise acquisition of mutations may transform cells to be CSCs, which have tumor initiating potential; (b) The intracellular bacteria may take up the host's DNAs and then develop into cancer cells by hybridizing the acquired DNAs with their own ones and expressing the hybrid genomes; (c) Cancer in adults may develop from embryonal rudiments that are produced in excess and that remain in the tissues of the fully

mature organs; (d) Cancer may arise in a cell that has the potential to divide and not be lost during normal tissue turnover; (e) Factors such as chemicals or viruses may induce dedifferentiation of mature adult cells to cause cancer; (f) Stem cells with gene mutations may acquire malignant phenotype and lead to cancer; (g) Progenitor cells may undergo transformation and become cancer cells (Yin et al., 2021).

1.2 BREAST CANCER

Breast cancer is the main cause of cancer death in women and the second most frequent cancer overall, after lung cancer. It is also the fifth most prevalent cause of cancer death. Breast cancer has a greater worldwide burden than all other cancers, and its incidence rates are rising (Hutchinson, 2010). Breast cancer is a complex condition with a variety of features that are sensitively linked to both genetic and environmental factors. It affects about one in ten women and is a major cause of death in developed nations (Kumar et al., 2013). Women over the age of 50 account for more than 80% of breast cancer cases. Family history is the third major risk factor, after gender and age. This denotes a high incidence of breast and ovarian cancer in the family as well as possible breast cancer in male relatives. According to Breast Cancer Care, a number of additional factors, such as obesity, excessive alcohol consumption, smoking, childlessness, first pregnancies after 30, menopause occurring later than the average age of 52, and periods starting before the age of 12, may also increase the risk (Breast cancer By Lynne Pearce). With an age-adjusted mortality rate of 12.7 per 100,000 women and a cancer incidence rate of 25.8 per 100,000 women, breast cancer is the most common cancer among Indian women. For incidence and mortality rates, data reports from the most recent national cancer registries were compared. It was shown that Delhi had the highest age-adjusted incidence rate of breast cancer at 41 per 100,000 women, followed by Chennai (37.9), Bangalore (34.4), and Thiruvananthapuram District (33.7). All of the PBCRs, including Bangalore (annual percentage change: 2.84%), Barshi (1.87%), Bhopal (2.00%), Chennai (2.44%), Delhi (1.44%), and Mumbai (1.42%), showed a statistically significant rise in age-adjusted rates over the study period (1982-2014). The mortality-to-incidence ratio was found to range from 8 in urban registries to 66 in rural ones (Malvia et al., 2017). Despite the fact that BC incidence rises with age, the pace of rise slows after menopause. This reflects the fact that BC is a hormone-

dependent disease and that post-menopausal women have lower levels of oestrogens and progestins in particular. Age at menarche, age at the first full-term pregnancy, and age at menopause are three life events that significantly affect the incidence of BC in women. Women who reach menarche at the age of 16 have a lifetime risk that is 50–60% lower than that of women who reach menarche at the age of 12. Similarly, whether it is caused naturally or by surgery, menopause that occurs 10 years before the typical menopausal age (52 years) lowers lifetime BC risk by about 35% (Libson, 2014).

Discovering up-to-date effective breast cancer prevention techniques and interventions is still difficult. The majority of women who do acquire breast cancer will be older than 50 when they are diagnosed, according to a big pooled analysis, despite the fact that they are at elevated risk due to having first-degree relatives with a history of the disease (Veronesi et al., 2005).

1.2.1 CAUSES OF DISEASE.

It was also shown that patients with normal body weight are less prone to cancer relapse, diabetes and cardiovascular diseases. Overweight and obesity aggravate the prognosis for the long-term follow-up period among women who received anti-cancer therapy (Kolak et al., 2017).

	Relative risk	High-risk group
Age	>10	Elderly individuals
Geographical location	5	Developed countries
Breast density	>5	Extensive dense breast tissue visible on mammogram
Age at menarche	3	Before age 11 years
Age at menopause	2	After age 54 years
Age at first full pregnancy	3	First child after age 40 years
Family history	≥2	Breast cancer in first-degree relative
Previous benign breast disease	4–5	Atypical hyperplasia
Cancer in other breast	>4	Previous breast cancer
Socioeconomic group	2	Groups I and II*
Body-mass index		
Premenopause	0.7	High body-mass index
Postmenopause	2	High body-mass index
Alcohol consumption	1.07	7% increase with every daily drink
Exposure to ionising radiation	3	Abnormal exposure to young girls after age 10 years
Breastfeeding and parity	Relative risk falls by 4.3% for every 12 months of breastfeeding in addition to a 7% reduction for every birth	Women who do not breastfeed
Use of exogenous hormones		
Oral contraceptives	1.2	Current users
Hormone-replacement therapy	1.66	Current users
Diethylstilbestrol	2	Use during pregnancy

Table 1.1- Risk factors in Breast cancer (Veronesi et al., 2005).

Race, ethnicity, family history of cancer, genetic characteristics, as well as modifiable exposures like increased alcohol consumption, physical inactivity, exogenous hormones, and specific female reproductive factors, are all well-established risk factors for breast cancer. Younger ages at menarche and parity, as well as longer ages for the first full-term pregnancy, may have a long-term impact on sex hormone levels, as well as other biological mechanisms, that may affect breast cancer risk (Coughlin, 2019). The risk of breast cancer in nulliparous women is higher than in multiparous women, according to medical literature. In a similar vein, nulliparous women who have their first child beyond the age of 30 have a slightly increased risk of breast cancer than women who often experience prolonged exposure to greater oestrogen levels. Menopause after the age of 55 and early menarche (before the age of 12) are also important risk factors. Over time, cyclical hormone levels can put women at risk for breast cancer. This may be due to the fact that pregnancy disrupts the normal cycle hormone levels, which cause breast cells to expand and divide in response to hormones like oestrogen (Durrani et al., 2018). Early breast cancer, locally progressed illness, and locoregional recurrence patients can all be cured. Multimodal therapy is common today. Surgery, radiation, and medication therapy are all part of it. Palliative care is used to treat patients with metastatic disease. Goals include symptom relief and extending survival (Wormann, 2017).

1.2.2 PREGNANCY – RELATED AND HORMONE-RELATED FACTORS.

The prevalence of pregnancy-associated breast cancer may be increasing owing to delayed childbearing, and despite its low incidence, breast cancer is the second most common cancer in pregnant women 10.7759/cureus.2941. The International Network on Cancer, Infertility, and Pregnancy (INCIP) conducted a large cohort study with 1170 patients over the course of 20 years. The results revealed that 67% of patients received cancer treatment while pregnant, and that the likelihood of doing so increased over time (Boere et al., 2022). Currently, there is strong evidence linking early age at first term birth to a lifetime risk reduction and increased risk of breast cancer. Even when age at first birth is taken into account, parity is linked to a long-term risk reduction; however, the additional, long-lasting protective effect of youthful age for future term births is not as great as that for the first term pregnancy; A nulliparous woman's risk is essentially equivalent to that of a 30-year-old woman

having her first term pregnancy; however, risk is momentarily elevated following a term pregnancy. Neither recognised spontaneous abortions nor induced abortions are linked to increased risk; and long duration of lactation has a small, additional reduction in risk after the age at pregnancy and number of term pregnancies are controlled (Veronesi et al., 2005).

1.3 COLORECTAL CANCER

Colorectal cancer (CRC) ranks second in terms of mortality (9.2%) and third in terms of recognition (6.1%). Rectal and colon cancer-related mortality are predicted to increase overall by 60% and 71.5%, respectively, by the year 2035 (Douaiher et al., 2017). Depending on the level of economic growth, these numbers may be different from one country to the next. As a result, the illness is commonly seen as a sign of the nation's socioeconomic growth (Bray et al., 2018). The increase in morbidity is also impacted by eating habits, lifestyle choices, and body fatness (Arnold et al., 2017). Physical activity has a protective impact, and this is supported by strong evidence. Increased consumption of red, processed meat, and alcoholic beverages increases the risk of developing the condition (Bray et al., 2018). In addition to better socioeconomic situations, the advancement of civilization and economic growth also leads to the "westernisation" of lifestyles, or changes in food patterns. This entails consuming more animal fats, processed meats, refined grains, and sweets, as well as less fruit, vegetables, and dietary fibre, and engaging in less physical activity. Such a lifestyle frequently contributes to overweight or obesity (Murphy et al., 2019). Obesity and overweight are linked to a higher risk of many modern diseases. The prognosis of CRC in men has been reported to be negatively impacted by visceral adiposity (Silva et al., 2020). Approximately 25% of the hereditary propensity is genetic. It is crucial to diagnose CRC at an early stage of development because the illness typically takes several to many years to manifest. Secondary prevention is also significant based on follow-up exams and nutrition prevention based on a balanced diet (Zaytseva, 2021).

1.3.1 EPIDEMIOLOGY

The second most frequent disease in both men and women, colorectal cancer is the third most prevalent cancer overall. In recent studies, there were more than 1.9 million new cases (Arnold et al., 2017). Nearly 935,000 cancer fatalities are thought

to have been caused by colorectal cancer, making it the second most prevalent type of cancer death (Sawicki et al., 2021). It accounts for 11% of all cancer diagnoses worldwide and is one of the tumours whose incidence is rising (Wong et al., 2021). Data from GLOBOCAN 2020 shows that there is a significant geographic variance in the incidence and mortality of CRC in different nations around the world fig1.4 (Sawicki et al., 2021)

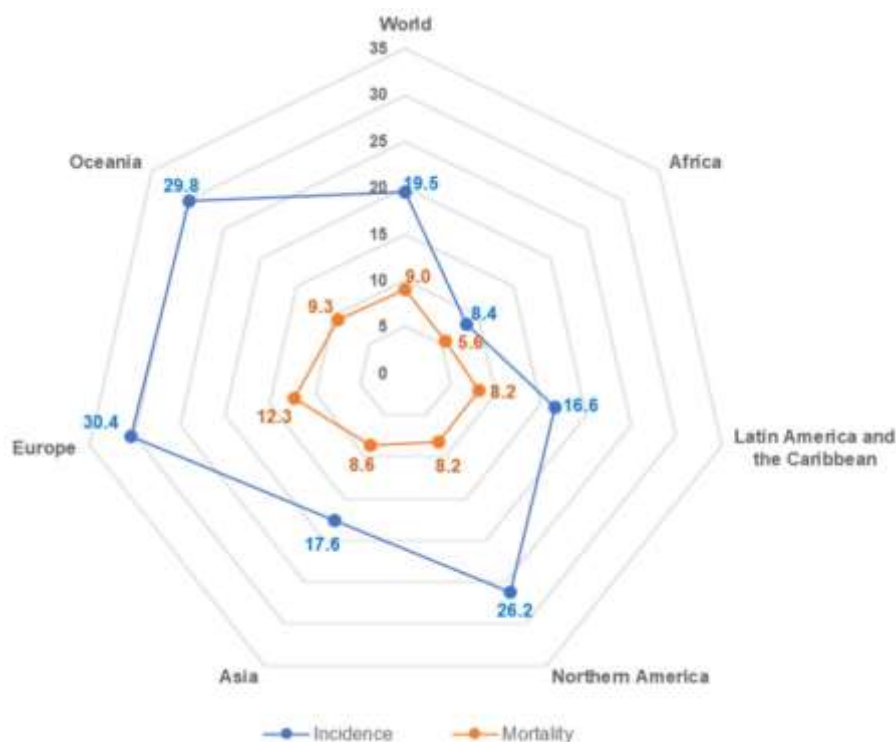


Figure 1.4 -Standardized incidence and mortality rates for CRC for both sexes in 2020, per 100,000 (Sawicki et al., 2021)

It has been established that nations with medium to high Human Development Indices (HDIs) that are embracing the "western" way of life see the largest increases in CRC incidence and mortality (Wong et al., 2021). The risk of colon cancer is greater in developed nations. Alcohol, cigarettes, red meat intake, sedentary lifestyles, and obesity are thought to be the main contributors to the rise in CRC (Arnold et al., 2017). So colorectal cancer is a condition that affects industrialised nations with a western way of life Cancer development is significantly impacted by factors that affect life expectancy, such as health-related behaviours (smoking,

obesity, and exercise) and social factors (education, income, and government spending on health). When creating measures to prevent and treat cancer, life expectancy levels must be taken into account (Chetty et al., 2016; Fidler et al., 2016). Numerous studies have drawn attention to the fact that the stage at which colorectal cancer is discovered affects how long patients survive, with later-stage diagnoses having a reduced chance of survival (Arnold et al., 2017; Bray et al., 2018; Wong et al., 2021). For colorectal malignancies that are detected early, the five-year survival rate is 90%, compared to 13% for those that are detected later. The cumulative risk of dying from colon cancer from ages 0-74 is 0.65% for males and 0.45% for women. The age-standardized (global) death rate for CRC is 8.9 per 100,000 for both sexes (Arnold et al., 2017). By 2030, there will be 1.1 million fatalities and more than 2.2 million new cases of CRC, an increase of 60% from recent years. Economic growth, or an economic transformation consisting of the shift from low- to medium-HDI nations and generational shifts in developed countries, will lead to such a huge increase. Numerous studies stresses that environmental changes like a more sedentary lifestyle, abnormal bone weight (obesity), intake of highly processed foods, alcohol, red meat consumption, and an increase in average life expectancy are also contributing factors (Wong et al., 2021).

1.3.2 RISK FACTORS

There are numerous factors that may contribute to the growth of colorectal cancer fig 1.5.

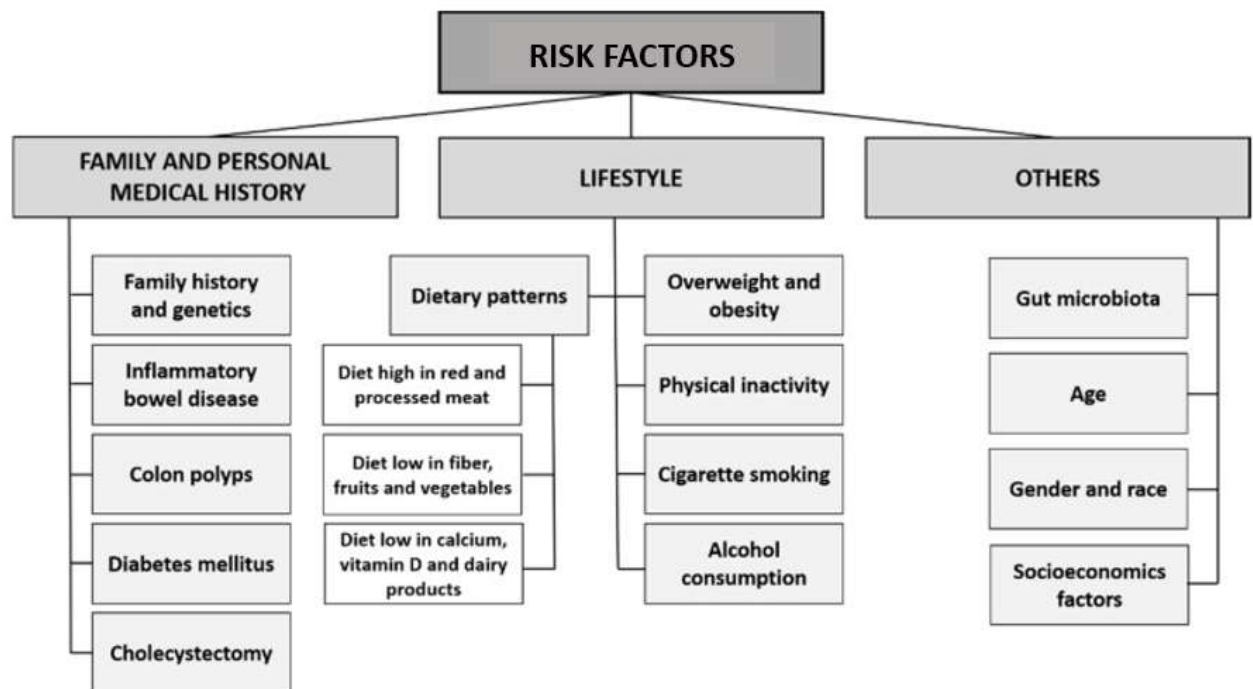


Figure 1.5- The main risk factors associated with colorectal cancer (Sawicki et al., 2021)

It has been shown that people are more likely to get CRC if they (or a family member) have ever had cancer, inflammatory bowel disease, cholecystectomy, colon polyps, or diabetes mellitus. Lifestyle factors are significant contributors to the development of CRC. According to the research, CRC risk factors include being overweight or obese, being physically inactive, smoking, drinking alcohol, and eating inappropriately (a diet high in red and processed meat and poor in fibre, fruits, vegetables, calcium, and nutritional items). In addition, colorectal cancer risk is known to be influenced by factors like age, gender, race, and socioeconomic position (Sawicki et al., 2021).

1.3.4 DRUGS FOR CANCER THERAPY

Classes or categories of drugs	Targets / mechanisms of repurposed drugs
Antidiabetics i. Metformin ii. Troglitazone, Pioglitazone, Rosiglitazone iii. Dapagliflozin, canagliflozin, empagliflozin Vildagliptin, sitagliptin, saxagliptin	Increases- AMP-activated protein kinase (AMPK) pathway Decreases- Insulin, insulin growth factor- (igf-1) pathway phosphatidylinositol-4,5 bisphosphat 3-kinase (PI3K)/AKT/mTOR pathway. Increase-Synthetic proliferator-activated receptor-gamma (PPAR- γ), AP-1, AKT, cyclin D1 Decrease-Sodium-glucose cotransporter-2 (SGLT2), AMPK pathway, WNT/ β -CATENIN PATHWAY, Decrease-MTOR, complex 1 of mitochondria, ATP Decrease-DDP4, glucagon-like peptide-1 (GLP-1). NF-kB
Antibiotics i. N-thiolated β -lactams, 4-alkylidene- β -lactams, and polyaromatic β -lactams ii. Penicillin G iii. Doxorubicin, daunorubicin, idarubicin, epirubicin, mitoxantrone, doxycycline, gemcitabine, duocarmycin SA iv. Landomycin E	Increases-DNA intercalation Decreases-Topoisomerase II Decreases-DNA synthesis Decreases-MMP11, STAT5A Decreases-Cancer stem cells Decreases-CD44

v. Salinomycin, Nigericin vi. Rapamycin	
Antifungal i. Intraconazole ii. Thiabendazole iii. Griseofulvin iv. Clotrimazole	Decreases-Hedgehog pathway Decreases-Angiogenesis Decreases- Microtubule dynamics, Increases-p53 Decreases- Actin polymerization, Decreases- glycolysis
Anti-inflammatory i. Aspirin ii. Ibuprofen iii. Diclofenac	Decreases- Cyclooxygenase (COX), NF-kB signaling, Decreases-CREB-binding protein/p300 acetyltransferase activity increases-AMPK path Increases-P75 neurotrophin receptor, decreases-heat shock protein 70 Decreases-Vascular endothelial growth factor, increases- p51, increases-Rb, increases-p21, decreases-Bcl-2

Table 1.2 - summarizes different antidiabetic drugs and their molecular targets (Fulda, 2015).

1.4 QUERCETIN

Quercetin (3,30,40,5,7-pentahydroxy-2-phenylchromen-4-one), a naturally occurring aglycone flavonoid, is derived from a variety of plant sources, including fruits, leaves, roots, and bark. It is a member of the polyphenol family with an flavonol core. The Latin acronym for quercetin is "Quercetum." Flavonols that are not produced by the body include quercetin in their group (Fulda, 2015). Several million chemicals originating from plants make up flavonoids, which all have the same phenylchromane structure. A variety of flavonoid subclasses, including flavonols, flavones, flavanones, catechins, isoflavones, anthocyanidins, dihydroflavonols, and chalcones, have been created as a result of this fundamental framework's flexibility in replacement structures. When compared to other flavonoids, flavonols and flavanols are the most widely present and highly plentiful in nature.

Since various epidemiological studies showing an unfavourable relationship between dietary consumption of flavonols and flavones and mortality were published, there has been increased interest in nutritional flavonoids. Five hydroxyl groups and three benzene rings make up the flavonoid's structure. There are two benzene rings (A and B rings) in quercetin [2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy- 4H-chromen-4-one], which are joined by a 3-carbon chain to form a closed pyran ring (C ring). There are five hydroxyl groups altogether, and any of them can be glycosylated to produce different quercetin glycoside forms when they attach to glucose, xylose, or rutin sugar (Magar and Sohng, 2020). The active groups in quercetin are the dihydroxy group between the A and B rings, the o-dihydroxy group of the B ring, and the 4-carbonyl of the C ring. These active phenolic hydroxyl groups and double bonds are largely responsible for quercetin's biological activity (Nguyen and Bhattacharya, 2022). Lower incidence and mortality from cancer and diseases connected to heart problems and cancer (Patel et al., 2018). It possesses special biological characteristics, such as anti-SARS-CoV-2, antioxidant, anti-inflammatory, anti-carcinogenic, antiviral, and antibacterial activity, that may increase disease resistance and general health (Mishra et al., 2020)

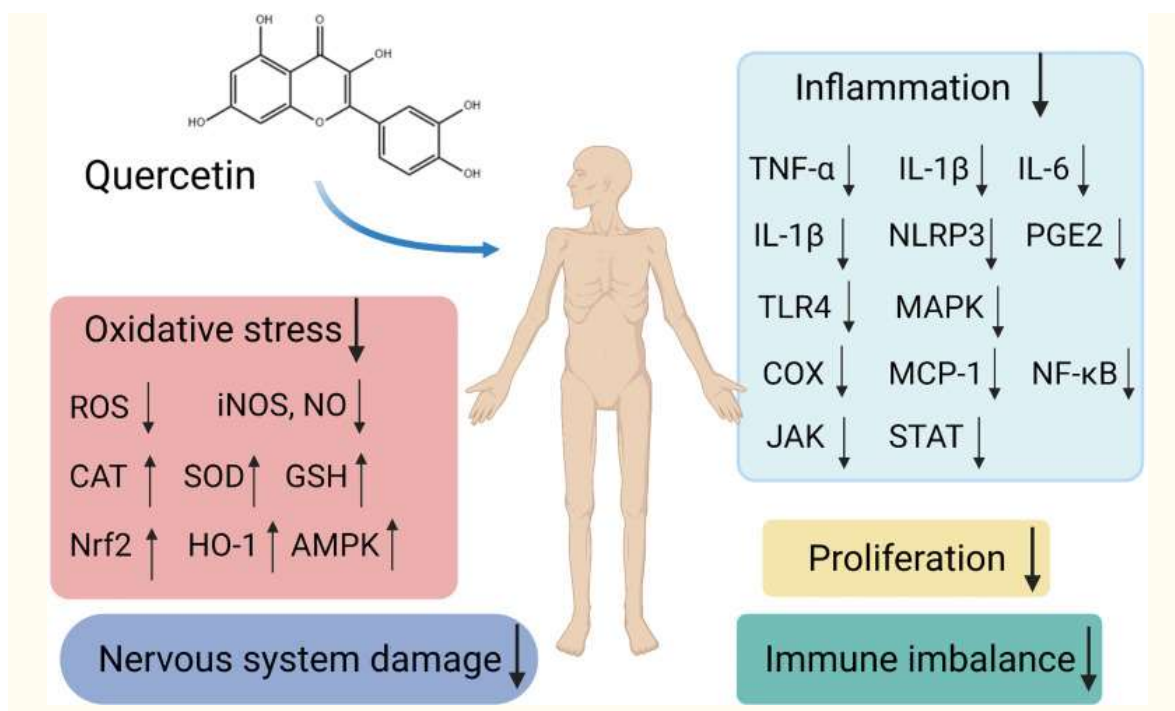


Figure 1.6- Schematic representation of different signaling pathways and targets by quercetin as a potential therapeutic strategy in autoimmune diseases (Shen et al., 2021)

Derivatives of quercetin can have a lipophilic or hydrophilic character depending on the type of substituents they include. The quercetin derivatives O-methyl, C-methyl, and prenyl are often lipophilic in nature. The glands found on the surfaces of leaves, flowers, and fruits synthesise them. By soaking the plant tissue in acetone⁴, they are easily separated. When heated at higher temperatures, quercetin structurally decomposes, releasing noxious odours and acrid smoke (Rana et al., 2022). Among these, onion is a key ingredient in many foods that contain quercetin. Onions contain quercetin in its glucoside form, specifically quercetin 4-O-b-D-glucoside (Q40 G) and quercetin 3,4-O-b-D-diglucoside (Q3,40 diG), with isorhamnetin 4-O-glucoside being present in lesser amounts.²⁰ These quercetin glucosides are present in yellow onion's edible flesh in amounts between 28 and 50 mg per 100 g (Terao et al., 2011). Quercetin has the chemical formula C₁₅H₁₀O₇ and is known by the nomenclature of the International Union of Pure and Applied Chemistry (IUPAC) as 3,3',4',5,7-pentahydroxyflavanone (or) 3,3',4',5,7-pentahydroxy-2-phenylchromen-4-one. The quercetin molecule in Fig.1.7 contains five hydroxyl groups at positions 3, 5, 7, 3', and 4'. These hydroxyl groups result in the formation

of and pentamethyl derivatives. Other names for it include quercetine, sophretin, and meletin (Rana et al., 2022).

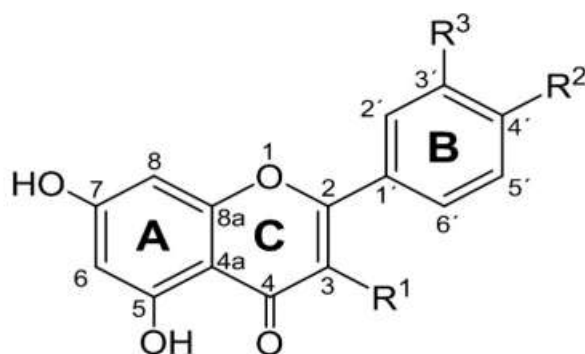


Figure 1.7- Chemical structure of Quercetin (Valentova et al., 2014).

The primary active location for antioxidant and reactive oxygen species (ROS) scavenging in the family of natural flavonoids is the B-ring (Tang et al., 2019). Quercetin capacity to scavenge free radicals is due to phenolic hydroxyl groups at the B-ring and the 3-position. Only conjugated metabolites of quercetin, which is typically present as a glycoside and is converted to glucuronide/sulfate conjugates after intestinal absorption, are consequently detectable in the bloodstream. Although metabolic conversion reduces its biological effects, increased b-glucuronidase activity during inflammation can produce active aglycone from the glucuronide conjugates (Murakami et al., 2008). Along with cancer cells, quercetin is utilised to treat cardiovascular disease, HIV, and malaria (Kumar et al., 2014). In several recent studies quercetin has reportedly attenuated rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, and systemic lupus erythematosus in humans or animal models (Shen et al., 2021). Additionally, quercetin has neuropharmacological protective properties against neurodegenerative mental illnesses like Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyloid peptide (Islam et al., 2021).

1.4.1 Antioxidant Activity

Oxidative stress is the term used to describe the pathophysiological reactions brought on by the body's excessive synthesis of highly reactive molecules like ROS and the dysregulation of the oxidant and antioxidant balance when exposed to

various damaging stimuli. Cardiomyocytes may experience apoptosis or necrosis, intracellular protein denaturation, lipid peroxidation, and other adverse effects as a result of oxidative stress (Li et al., 2021). Given that quercetin is an effective ROS scavenger, it guards the body from oxidative stress. Quercetin also controls the body's glutathione (GSH) level and maintains the oxidative equilibrium, making it a powerful antioxidant (Xu et al., 2019). With the pretreatment of quercetin, it has been observed that superoxide dismutase (SOD), catalase (CAT), and GSH expression levels are raised. Numerous investigations have demonstrated that quercetin directly interacts with DNA and covalently binds to DNA (Xu et al., 2019). To prevent lipid oxidation, quercetin can be utilised as a safe dietary polyphenol (Liu et al., 2021). In addition to improving M2 macrophage polarisation and endogenous antioxidant expression in both macrophages and microglia, quercetin significantly decreased the production of ROS in the microglia (Tsai et al., 2021). According to reports, quercetin's antioxidant activity is unaffected by the esterification step. In fish oil, quercetin significantly reduced aldehyde production and unsaturated fatty acid oxidation (Oh et al., 2021). Derivatives of quercetin may therefore be helpful as potential antioxidants in foods and biological systems. As a result, quercetin dietary supplements can help quail by reducing oxidative stress, metabolic alterations, and apoptosis. Quercetin has also been shown to be efficient at inhibiting UVB-induced intracellular ROS formation, hence protecting mitochondria. UVB stimulates the production of ROS inside HaCaT cells (Zhu et al., 2017).

1.4.2 ANTICANCER ACTIVITY

1.4.2.1 Antitumour

Cancer is a highly lethal condition. Given its current state, it is vital to research a type of medicine that is less toxic, has fewer side effects, and is an effective adjuvant therapy or treatment for cancer. Several pathways, several connections, and various targets are involved in the formation and incidence of tumours. When used at a suitable amount, the natural substance quercetin inhibits the growth of tumours in a variety of ways while being harmless (Rauf et al., 2018). The primary methods that quercetin fights cancer include through preventing cancer cell growth, triggering apoptosis in cancer cells, encouraging autophagy in cancer cells, controlling signalling pathways, preventing cancer cell invasion and metastasis, improving

chemotherapy sensitivity, and overcoming drug resistance (Tang et al., 2020)). Treatment with quercetin in combination with a growth factor midkine knockdown method may target CD44+/CD133+ cells and encourage their clearance, limiting cancer relapse (Erdogan et al., 2018).

1.4.2.2 Cancer Prevention

Cancer chemoprevention is a strategy for preventing the disease that entails using one or more natural or synthetic medications over an extended period of time to slow or stop the progression of cancer before it progresses to an aggressive form. Quercetin specifically activates apoptosis in cancer cells without impairing normal cell growth, making it an ideal therapy option for chemoprevention of cancer (Rather and Bhagat, 2020). Quercetin is regarded as a supplemental or alternative medicine for the prevention and treatment of many malignancies, according to the available studies. The growth and apoptosis of the human breast cancer cell line MCF7 were markedly inhibited at the proper molar ratios of quercetin and curcumin (Mansourizadeh et al., 2020). In addition, quercetin reduces levels of PKM2, GLUT1, and LDHA, which may play a key role in the prevention of breast cancer by preventing glucose uptake, lactic acid generation, and cancer cell motility. Quercetin suppresses the mobility of breast cancer by suppressing glycolysis through Akt-mTOR pathway mediated autophagy induction. In addition, quercetin reduces levels of PKM2, GLUT1, and LDHA, which may play a key role in the prevention of breast cancer by preventing glucose uptake, lactic acid generation, and cancer cell motility (Jia et al., 2018).

1.4.2.3 Anticancer Agent

By controlling signal molecules and obstructing cell cycle, quercetin can improve the overall efficacy and safety of anticancer medicines while reducing their dosage. Cervical cancer cells are inhibited synergistically by quercetin and cisplatin (Xu et al., 2021). By reducing the expression of MMP-2, ezrin, METTL3, and P-Gp in cancer cells and diminishing their ability to proliferate, migrate, and invade as well as increase apoptosis, quercetin may improve the anticancer effects of cisplatin (Sunogrot et al., 2019). The plant polyphenol quercetin was functionalized with polyethylene glycol and loaded with hydrophilic anticancer curcumin for drug delivery applications of cancer cell lines with stronger antiproliferative capabilities. The

majority of quercetin-based nanomaterials were found to be spherical and to have good solubility in aqueous solutions, which unquestionably increases their anticancer potency. In addition, quercetin's effects on glucose metabolism and cellular energy production contribute to its effects on cancer cells' decreased viability, metastasis inhibition, and induction of apoptosis (Reyes-Farias and Carrasco-Pozo, 2019). Numerous in vivo and in vitro studies have demonstrated the potent effects of quercetin in triggering apoptosis, preventing metastasis, and controlling cell cycle and tumour angiogenesis (Table 1.3)

Cancer types	Study design	Quercetin/ derivative/ sources	Study model s	Effects/molec ular mechanisms
Prostate cancer	In vitro	Quercetin	PC3 and LNCaP cells	Increase cyt c, casp 3,casp 8, bax, Bcl- 2,p21Cip1,p27 Kip1, and p53 decrease p38,NF-kB, and survivin protein level and increase the PTEN expression
Liver cancer	In vitro	Quercetin	KIM-1, YN-1, KYN-2, YN-3, HAK-1A, HAK-5, HAK- 6	Inhibit apoptosis and cell cycle arrest
Hepatocellul ar carcinoma	In vitro	Quercetin	HepG2, HuH- 6, and HepT1 cells.	Affect the mitochondrial integrity and disturb the ROS

			Human hepatoblastoma tissues	homeostasis depending, activate Raf/MEK, AKT, and NF-kB pathway
Prostate cancer	In vitro	Quercetin	LM3 cells, Nude mice	Inhibit the growth of a panel of 9 cancer cell lines

Table 1.3- Cancer types with its respective mechanism (Wang et al., 2022).

The medicinal potential of numerous phytochemicals extracted from various natural sources has been theorised; quercetin is one of the most well-known and medicines with biological activity is one of them. Quercetin is anticipated to develop into a brand-new medication that may both prevent and treat different disorders through ongoing research. Numerous in vivo and in vitro studies have demonstrated quercetin's positive pharmacological properties, which include anti-SARS-CoV-2, antioxidant, anti-cancer, anti-aging, antiviral, and anti-inflammatory properties, as well as potential mechanisms that may be associated to these properties (Reyes-Farias and Carrasco-Pozo, 2019).

1.5 SILVER NANOHYDROXYAPATITE

Hydroxyapatite (HAp, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), which is a type of bioceramic that is chemically identical to the mineralized stage of real bone and teeth and falls under the category of calcium orthophosphate (Bee et al., 2021). Hydroxyapatite nanoparticles (HAp NPs), in addition to their well-known uses in bone tissue engineering, have shown significant potential for enhanced cancer therapy. In comparison to traditional chemotherapeutic methods, HAp NPs' chemical structure provides good opportunities for loading and delivering a wide variety of anticancer medicines in a sustained, extended, and targeted way. Another strategy for delivering advanced anticancer effects, such as the capacity to inhibit the growth and metastasis of cancer cells by activating particular cell signalling pathways, is

the incorporation of specific therapeutic elements into the basic composition of HAp NPs. This strategy can be used alone or in combination with drug release. By using various surface modification techniques, HAp NPs can readily be transformed into smart anticancer agents that make it easier to target and destroy cancer (Kargozar et al., 2022). Since nano-hydroxyapatite (nHAp) is a type of nanomaterial, it has the tiny size effect of small crystal grain width, big interface, high surface free energy and binding energy, as well as the special physical and chemical characteristics of nanomaterials including the macro quantum tunnelling effect. Anti-tumor medications are also delivered using nano-hydroxyapatite as a drug carrier. Nano-hydroxyapatite porous microspheres have many benefits as an anti-tumor drug carrier, including a large specific surface area, high drug load, good body compatibility, pH-responsive degradation, etc. They have been used in tumour detection, anti-tumor drug delivery, and other fields. Hydroxyapatite with porous surface structure and -OH groups may serve as an ideal candidate drug carrier for the transport of a variety of pharmaceutical compounds because of its bioactivity, biocompatibility, stability, and nontoxic qualities (Zhang et al., 2010). It should be highlighted that physicochemical characteristics including particle size, crystallinity level, surface area, shape, and surface charge mostly determine how HAp reacts biologically ((Kermani et al., 2020), (Kermani et al., 2021a)). For instance, a high dissolution rate will be caused by a low crystallinity level. The loss or leaching of therapeutic substances by the theranostic device may not always be advantageous. Additionally, HAp with a variety of characteristics and varied solubilities in the biological environment may enhance bone formation or inhibit the growth of cells and tissues in situations where ion homeostasis is dysregulated (Lara-Ochoa et al., 2021). HAp was initially suggested for use in hard tissue engineering applications in medicine due to its superior physico-chemical, mechanical, and biological properties, as has been discussed elsewhere (Dorozhkin, 2013). The invasion of skeletal tissues by cancer cells—which are primarily made up of HAp—indicates that there is a significant relationship between HAp and cancer cells (Beik et al., 2016). According to earlier investigations, HAp's chemical structure offers outstanding opportunities for ion exchange in both its anionic (PO_4 , OH) and cationic (Ca) locations (Awasthi et al., 2021). A variety of anticancer medications and compounds could be loaded onto HAp NPs and released over time as part of tailored cancer therapy strategies (Abbasi Aval et al., 2016). It is widely known that

by reducing systemic toxicity and raising the local concentration of an anticancer agent, targeted therapy improves the therapeutic effects of anticancer medicines both in vitro and in vivo. The conjugation and grafting of particular micro- and macro-molecules onto the particle surface has enabled the targeting of HAp towards tumour cells, resulting in increased HAp uptake by tumour cells ((Ansari et al., 2020) (AbouAitah et al., 2020)). The value of HAp nanoparticles in multimodal molecular imaging has been suggested over time. In this way, magnetic resonance (MR) and nuclear imaging have previously been approved uses for superparamagnetic HAp NPs as in vivo agents (Adamiano et al., 2018). Additionally, HAp NPs could be functionalized by various biocompatible substances (such as polymers) in both their pristine and ion-doped forms. This would allow for improved bioimaging techniques, such as cellular imaging and non-invasive and quantitative visualisation of molecular processes (Zhou et al., 2020).

1.5.1 Hallmarks of Cancer: A Call for Theranostic Nanoparticles

Six major hallmarks of cancer were first introduced by Hanahan and Weinberg in (Hanahan and Weinberg, 2000) which are (I) sustaining cellular proliferative signaling; (II) insensitivity to anti-growth cellular signals; (III) resistance to cellular apoptosis; (IV) cellular replicative immortality; (V) stimulated angiogenesis; and (VI) tissue cancer cell invasion and metastasis (Hanahan and Weinberg, 2011). In addition, two more emerging hallmarks to those mentioned above were introduced: changing cellular energy metabolism and avoiding immune system damage (Hanahan and Weinberg, 2011). In fact, it has been determined that each of these processes plays a significant role in the astonishing variety of neoplastic disorders that disrupt genomic stability (Kargozar et al., 2022). NPs, such as HAp nanostructures, have previously been proposed to simultaneously target some of the characteristics of cancer and to transport and release several cytotoxic chemicals in a secure and efficient manner (VanDyke et al., 2016). For instance, HAp NPs can reduce the FAK/PI3K/Akt cell signalling pathway both in vitro and in vivo, which inhibits cancer cell proliferation, migration, and invasion (Wang et al., 2020). Apoptosis in cancer cells brought on by oxidative stress can also be brought on by HAp NPs through lysosomal and mitochondrial-dependent mechanisms, including caspase 3 (VanDyke et al., 2016). However, there are very few studies in

the literature that concentrate on employing nano-sized HAp particles, either in the pristine or in the functionalized and drug-loaded kinds, to target and image the many hallmarks of cancer (Kargozar et al., 2022).

1.5.2 HAp Structure Fits Therapeutic Applications

The space groups of the hexagonal and monoclinic systems in which HAp can crystallise are P6₃/m and P2₁/b, respectively (Elliott et al., 1973). The hexagonal systems' lattice parameters for HAp are $a = b = 9.432^\circ$, $c = 6.881^\circ$, and $\gamma = 120^\circ$ (doi: 10.1002/wnan.1504) while the lattice parameters of the monoclinic structure of HAp are $a = b/2 = 9.421 \text{ \AA}$, $c = 6.881 \text{ \AA}$, and $\gamma = 120^\circ$ (Tello et al., 2020). In the hexagonal structure of HAp, neighbouring hydroxyl (OH) groups face one another in the opposite direction, whereas in the monoclinic structure, all of the OH groups in a given column face the same

Incorporating therapeutic ions and heat treatments at high temperature are two of the most popular methods for creating structural flaws in HAp during its (Bystrov et al., 2021). The flaws in HAp can be divided into many types, such as impurities (impurities in carbonated groups are frequently found), interstitial protons, oxygen vacancies (O removal from O-H or PO₄ groups), and vacancies in O-H groups (Kermani et al., 2021b). HAp's physico-chemical characteristics, such as the band-gap, surface charge, and area, as well as the ion release/uptake, may be altered by intentionally made flaws, which could enhance the biological behaviour (for example, enhance osteogenesis. Due to its ease of internalisation into cancer cells and capacity to promote the targeted administration of anticancer agents, the use of nanoscaled HAp has drawn a lot of interest in cancer diagnostic methods (Ren et al., 2020). HAp NPs with a specific microstructure have so far been prepared using a variety of chemical techniques. Five categories could be used to categorise all of the synthesis routes: (I) dry methods (solid-state and mechanochemical routes); (II) wet methods (chemical precipitation, hydrolysis, sol-gel, hydrothermal, emulsion, and sonochemical routes); (III) high-temperature processes (combustion and pyrolysis methods); (IV) methods based on biogenic sources (biogenic wastes); and (V) combination procedures. The aforementioned techniques can be used to create HAp nanostructures of various sizes and shapes, including as spheres, rods, needles, flakes, flowers, mesoporous spheres, bowknots, dumbbells, etc (Sadat-

Shojai et al., 2013). It should be noted that HAp NPs' shape, crystallinity, and size are some of the most important aspects impacting their anticancer properties (Wu et al., 2019). The aforementioned characteristics had a significant impact on the anionic and cationic sites of HAp that contained hydroxyl, amino, and carboxyl groups as well as calcium and phosphorus ions. The interaction of HAp with a biomolecule is greatly influenced by the positive and negative surface charges of HAp, which are dependent on its physicochemical characteristics (Kargozar et al., 2022). HAp NP structural alterations offer great opportunities for the production of self-activated fluorescent compounds, which are excellent intracellular imaging tools for both prokaryotic and eukaryotic cells. These substances are also therapeutic approaches (Deshmukh et al., 2016). HAp NPs can be used for cancer cell imaging in addition to structurally modified HAp nanostructures through a variety of well-established methods, such as (I) combining them with organic fluorophores, (II) doping them with different lanthanide ions, and (III) creating composites with other inorganic nanomaterials (such as carbon-quantum and chalcogenide-quantum dots) (Guo et al., 2021).

1.5.3 HAp Nanostructures for the Delivery of Anticancer Drugs

There are various advantages to using nanostructured HAp as a drug carrier in cancer theranostic applications (Saber-Samandari et al., 2016). Following is a summary of these benefits: (I) HAp could be prepared with relatively similar properties (e.g., chemistry, crystalline structure, and size) to the major constituents of the targeted tissues (especially bone); (II) HAp alone is relatively stable under variations of the solution/environment (e.g., pH, temperature)—unless produced in the form of NPs—and does not swell or change porosity, preventing the burst release of drugs; (III) HAp can be surface-functionalized with both positively and negatively charged molecules; and (IV) HAp might be tailored as NPs with promising electrical, mechanical, magnetic, and optical properties via doping with different elements (Mondal et al., 2018). In order to manage tumours connected to both hard and soft tissues, a variety of natural and synthetic anticancer drugs have been loaded onto and delivered using HAp NPs (Govindan et al., 2017). Mesoporous HAp NPs are a promising class of drug delivery systems that can be used to transport a variety of anticancer cargos, including as chemicals, small compounds, and generic medications. These systems have outstanding drug adsorption, storage, and

release capabilities, which make them promising in cancer therapy techniques (Meshkini and Oveisi, 2017).

Due to their wide-ranging antibacterial properties against microorganisms, low bacterial resistance, and relatively low cytotoxicity towards mammalian cells, silver (Ag) compounds and silver nanoparticles (AgNPs) have attracted significant consideration as effective antibacterial agents over the past ten years. Because of their well-known antibacterial qualities, silver nanoparticles (AgNPs) are employed in numerous healthcare items, medications, and medical equipment. Several groups recently have discovered that different malignant cell lines are affected by the anti-tumor actions of silver ions. Kovacs et al. discovered that P53-expressing U2Os and P53-deficient Saos-2 osteosarcoma cells may both be employed to inhibit the formation of 5- and 35-nm sized AgNPs (Veerla et al., 2019).

In the present work, we aimed to further prepare an antibacterial HAp/AgNPs nanocomposite via the chemical reduction and calcination methods. In this context, the HAp that is commercially available will serve as a solid support for the decoration and deposition of AgNPs to form HAp/AgNPs nanocomposite. The chemical reduction route employed was based on the use of N,N-dimethylformamide (DMF) and poly(vinyl acetate) (PVA) as reducing and stabilizing agents, respectively, to induce the formation and deposition of AgNPs on the HAp from Ag salt precursor.

As far as we are concerned, very little research conducted related to the incorporation of HAp with AgNPs in enhancing anticancer properties, especially using Hap powder. Furthermore, there is a scarce information reported regarding the Ag⁺ ion release in long-term studies. Indeed, the scrutiny of Ag⁺ ion release is crucial to ensure the released ion concentration is below the cytotoxic level over a sustain period of time. Herein, the physico-chemical, Ag⁺ ion release behavior, anticancer and bioactivity properties of the resulting nanocomposites were investigated elaborately with respect to Ag salt concentration used. Moreover, the formation mechanism of HAp/AgNPs nanocomposite was also proposed and elucidated in this study.

CHAPTER-2

LITERATURE SURVEY

2.1 CANCER

With 410 million deaths from cancer worldwide each year, it is one of the main causes of mortality. Current cancer treatments include surgery, radiation, and the use of chemotherapeutic medications, which frequently cause toxicity in patients and the death of healthy cells. So, scientists are trying to figure out how to only get rid of malignant cells. (Hausman, 2019) in his study proposed that the primary barrier to an effective cancer treatment is the heterogeneity of malignant cells within the tumour. The treatment of different malignancies can be enhanced, nevertheless, by a greater understanding of the molecular basis of tumours and the development of novel diagnostic technologies. Therefore, it is crucial that we investigate epigenetic modifications, the gene expression of malignant cells, and use techniques that allow us to reverse or lessen these changes. As Anya Plutynski puts it in *Explaining Cancer* (2018): “Cancer may be viewed as akin to a car breaking down, a process of natural selection, an infectious disease . . . a process of development, and even the growth of an ecological community”. The most challenging characteristics of cancer—metastasis, recurrence, and therapeutic resistance—have recently been the subject of a convergence of theories that has produced a cohesive theoretical framework. The new paradigm of cellular differentiation, which was previously thought to be a unidirectional process but is now understood to be a flexible process in which cancer cells can dedifferentiate into more primitive, stem-like phenotypes, forms the core of this framework. Both inherent biochemical mechanisms and reciprocal environmental cues involving non-cancerous cells connected to cancer regulate this plasticity. Such flexible phenotypic changes may have an impact on malignancies' discontinuous behaviour, in which some tumours go untreated for months or even years before relapsing and wreaking havoc. Wael M et.al 2013 also said that metastasis, relapse, and therapy resistance are three of the most difficult elements of cancer. The new paradigm of cellular

differentiation, which was previously thought to be a unidirectional process but is now understood to be a flexible process in which cancer cells can dedifferentiate into more primitive, stem-like phenotypes, forms the core of this framework. Both inherent biochemical mechanisms and reciprocal environmental cues involving non-cancerous cells linked to cancer regulate this plasticity. Such flexible phenotypic changes may have an impact on malignancies' discontinuous behaviour, in which some tumours go untreated for months or even years before relapsing and wreaking havoc. Somatic cells in the human body with genetic mutations accumulate to form cancers. Marc J et al 2019 proposed that these mutations alter the cells' phenotype, enabling them to evade the homeostatic control that preserves a normal cell count. According to evolutionary biology, the conversion of healthy cells into cancerous cells is an example of evolution at action. Evolution caused by adaptation to changes in the cancer's environment, persists throughout cancer growth, progression, treatment resistance, and disease relapse, and intratumor heterogeneity is an inevitable byproduct of this evolutionary process. The use of genomics to characterise tumour evolution allows for the quantitative measurement of evolving clones over both space and time.

2.2 BREAST CANCER

Breast cancer remains a serious health issue despite significant advancements in the field of cancer research, and is currently a high focus for biomedical research. Zoi Anastasiadi et al, 2016 said the most frequent cancer among women worldwide is breast cancer, and both its incidence and fatality rates are predicted to rise sharply in the coming years. Recent cases of breast cancer in young women have caught the attention of researchers. According to recent data, breast cancer is clearly the top cause of cancer-related fatalities in women under the age of 45. The basic characteristics of this form of cancer appear to be potentially aggressive and highly heterogeneous. The 'complex' biology of this particular cancer type is still unknown and understudied, and therapy techniques, guidelines, and options are not based on age. Shai Libson et al 2014, Breast cancer detection and diagnosis abilities have significantly increased. The underlying biological mechanisms are now being more heavily considered in treatment decisions that were formerly mostly centred on the

anatomic extent of the disease. Breast cancer is now understood to be a heterogeneous disease made up of various biological processes thanks to the development of gene array technology. The ability to predict chemotherapy response is made possible by subtypes and genetic profiling. The oncoplastic method allows for broad excisions without affecting the breast's natural contour, and breast conservation has become the accepted standard of treatment. The conventional approach to stage the axilla has been superseded by sentinel lymph node biopsy, sparing many patients the further morbidity of axillary dissection. Kolak A et al 2017 said High-fat diets, alcohol use, and a lack of physical activity are just a few examples of lifestyle and environmental factors that have been shown to influence the development of mammary gland cancer; eliminating these factors (primary prevention) may help reduce morbidity and death. second line of defence, Including diagnostic tools that aid in the early diagnosis of cancers or lesions predisposing to tumours (such as mammography, ultrasonography, magnetic resonance imaging, breast self-examination, and contemporary, more accurate imaging techniques).

2.3 COLORECTAL CANCER

Sigurdis Haraldsdottir et al,2014 showed the incidence of colorectal cancer rises with age, making it the third most frequent cancer in the Western Hemisphere. The majority of colorectal tumours are localised, whether or not lymph node metastases are present. Up to 20% of individuals had metastatic disease upon presentation, most frequently to the liver. The only curative treatment for locally advanced colorectal cancer is surgery, and patients with lymph node metastases are typically advised to have adjuvant chemotherapy. The three main treatments for rectal cancer are surgery, radiation therapy, and chemotherapy. Surgery can save some patients with recurring and metastatic illness, but chemotherapy is still the backbone of treatment for advanced colorectal cancer. In recent years, significant advancements in the treatment of metastatic colorectal cancer have been noted. Kannan Thanikachalam et al,2019 also said that While successful cancer screening programmes have reduced the incidence and mortality rate of colorectal cancer, there has been a rise in the proportion of young people receiving a colon cancer

diagnosis for currently unknown reasons. Although nutrition may have both a causative and preventive impact in the pathogenesis of colon cancer, environmental and genetic variables also play a significant role in this disease. Jürgen Weitz et al, 2005 said around 492000 patients globally pass away from colorectal cancer each year, which affects over 945000 people annually. This particular type of cancer usually arises incidentally, in connection with genetic cancer syndromes, or as a result of inflammatory bowel illnesses. All of these causes have screening and prevention strategies that should be made more widely known. Colorectal cancer develops through an adenoma-carcinoma sequence, and the underlying molecular alterations have essentially been discovered. The patient, the treatment, and the cancer all influence the prognosis, and one of the key predictors of result is the treatment team's level of skill. The development of targeted therapy alternatives, which are currently being explored in clinical trials, is the result of new knowledge regarding the molecular causes of this cancer.

2.4 QUERCETIN

Solaimuthu Balakrishnan et al, 2016 conveyed that the proliferation, survival, and metastasis of breast cancer cells are significantly influenced by epidermal growth factor. A bioactive flavonoid called quercetin has been proven to have anticarcinogenic properties against many malignancies, including breast cancer. Quercetin is a plant polyphenol from the flavonoid group that plays a fundamental role in controlling homeostasis due to its potent antioxidant properties. Fahimeh Aghapour et al 2018, described a simple method for the synthesis of quercetin nanoparticles. The quercetin nanoparticles had an average diameter of 82 nm and prominent yellow emission under UV irradiation. Therefore used an in vitro model treated with quercetin and quercetin nanoparticles to investigate the effects of quercetin nanoparticles on MCF-7 breast cancer cell line. Hayde Vergara-Castañeda et al 2015 worked to formulate biofunctional hybrid materials (HMs) with quercetin (QC) and silica particles (SiPs) by simple methods such as sol-gel and QC conjugation. Physicochemical characterization included particle size, zeta potential. Akram Bardestani et al 2021 Neuroprotective effects of quercetin (QC) have been proven due to its antioxidant and anti-inflammatory properties. However,

poor solubility and low bioavailability of QC have also led researchers to make various QC-involved nanoparticles to overcome these limitations. We wondered how high doses or prolonged treatment with quercetin conjugated superparamagnetic iron oxide nanoparticles (QCSPIONs) could improve cognitive dysfunction and promote neurogenesis without any toxicity. It can be explained that the QC inhibits protein aggregation and acts against iron overload via iron-chelating activity, iron homeostasis genes regulation, radical scavenging, and attenuation of Fenton/Haber–Weiss reaction.

2.5 SILVER HYDROXYAPATITE

Biomaterials are often used in orthopedic surgery like cavity fillings. Juan J Martinez-Sanmiguel et al, 2019 said related complications often require long-term systemic antibiotics, device removal, and extended rehabilitation. Hydroxyapatite/silver (HA/Ag) composites have been proposed as implantation biomaterials owing to the osteogenic properties of hydroxyapatite and to the antimicrobial efficiency of silver. Nevertheless, higher silver concentrations induce cytotoxic effects. The aim of the study was to synthesize and characterize HA/Ag nanocomposites that will allow us to use lower concentrations of silver nanoparticles with better antimicrobial efficiency and anti-inflammatory properties. Umit Erdem et al, 2019 showed the result of biological, physical and chemical effects and may cause toxicity. Therefore, it is important to determine whether the presence of Ag (I) has a toxic effect. In this study, Ag (I)-doped nHAp was synthesized by precipitation method and tried to determine the release values as a function of time compared to the doping rate by using the ICP-OES. Also, the products we produce in simulated body fluid were kept for retention periods of 4–20 weeks to determine degradation percentages. A cytotoxicity study was performed to observe the toxic effect that may be caused by possible Ag (I) release. According to the analysis, the release values in all products were observed in ppb level. And it is concluded that the materials produced are not degraded. . Takema Nakashima et al., has been reported to Silver-containing hydroxyapatite coating has been reported to have good biocompatibility and low toxicity. Vijay Bhooshan Kumar et al., reported the osteogenic potential of silver (Ag), gold (Au), or silver–gold doped hydroxyapatite nanoparticles (Ag-Au-HA) in

zebrafish (ZF) jawbone regeneration (JBR) model. The hydroxyapatite (HA, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), Ag-HA, Au-HA, and Ag-Au-HA nanomaterials were synthesized by the co-precipitation procedure. The surface structures of Ag-HA, Au-HA, HA, and Ag-Au-HA were analysed by scanning electron microscopy, transmission-electron microscopy (TEM), x-ray diffraction, Fourier transform infrared (FTIR), UV-vis, energy dispersive x-ray spectroscopy (EDS), elemental mapping, and laser fluorescent spectroscopy.

CHAPTER-3

AIM AND SCOPE

3.1 AIM

To study the effect of Quercetin loaded nanoporous Silver Hydroxyapatite nanoparticle which is suitable as a nanocarrier for the anti-proliferation activity against various cancer cell lines.

3.2 SCOPE

- ✚ Quercetin has been reported as one of the effective anticancer drugs, however quercetin cancer therapy still carries some drawbacks, such as limited applications for killing only few types of cancer cells, poor specificity and associated toxicity.
- ✚ To overcome these disadvantages drug carrier methods that emphasize biological materials have been developed in this study and applied to optimize various cancer treatments.
- ✚ In this study we synthesized biocompatible inorganic material, hydroxyapatite which was applied as a nanocarrier to deliver anticancer drug effectively into the cancer cells and enhance the stability and sustained release of the anticancer drug molecules to various cancer cells
- ✚ To study the substantial role of Ag-Hydroxyapatite concentrations on the formations of core-shell nanoparticles and loading of quercetin on Ag-HYP by conjugation method
- ✚ Further confirmation of Ag-HYP with quercetin was performed by different characterization techniques such as UV, XRD, FTIR and FESEM which confirms the quercetin conjugation with Ag-HYP.
- ✚ Enhanced antioxidant and anticancer effect of Quercetin was studied
- ✚ To perform the cytotoxic effect of Quercetin on vero cells and cancer cells lines, with respect to the proliferation potential.
- ✚ The improvement in the solubility of Quercetin by loading onto mesoporous Ag nanocomposite particles loaded Quercetin compared with the Quercetin drug alone

CHAPTER -4

MATERIALS AND METHOD

4.1 MATERIALS

Quercetin powder from, PVA ($M_w = 190,000$) and silver nitrate (AgNO_3), Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS), MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide), Dimethyl sulfoxide (DMSO), Dimethylformamide (DMF), Sodium hydroxide (NaOH) and Phosphate buffered saline (PBS). Throughout the experiment, Sterile distilled water was used to prepare aqueous solution. All chemicals were used without further purification.

4.2 SYNTHESIS AND CHARACTERIZATION OF SILVER HYDROXYAPATITE NANOPARTICLES.

4.2.1 SYNTHESIS OF AgHAp NANOPARTICLES

Commercially available HAp powder is taken and Deposition of AgNPs onto the HAp surface was carried out based on chemical reduction of Ag^+ ions, where DMF and PVA serve as reducing and stabilizing agents, respectively. Typically, AgNO_3 with different concentration (i.e., 1, 3, and 5 wt.%) were dissolved in 5 mL of DMF under constant stirring for 3 h to form metallic Ag solution. Subsequently, 2.5 g of as-prepared HAp and 2.5 g of PVA were added into 50 mL of DMF and mechanically stirred for 1 h. Thereafter, the Ag solution was added dropwise into the mixture and allowed to stir overnight. It should be noted that all reaction was conducted under dark condition to minimize photo-activation of AgNO_3 . During the reduction reaction, the milky white slurry of HAp gradually transform into dark brown or gray suspension. After the completion of the reaction, the products were collected through filtration, washed with distilled water for several times, and dried in oven at 50°C . Finally, the dried powders were calcined at 600°C with soaking time of 1 h in order to remove the residual PVA. The prepared samples were denoted as

HAp/AgNPs-x nanocomposite, where x refer to the concentration of AgNO₃ added (Bee et al., 2021).

4.2.2 CONJUGATION OF SILVER NANOHYDROXYAPATITE WITH QUERCETIN

Quercetin was conjugated with AgHAp by adding them in 1:1 ratio in 1ml sterile distilled water. This solution was vortexed thoroughly to obtain the colloidal suspension, The nanoparticle colloidal suspension was ultrasonicated for 10minutes and stored at 4°C until use.

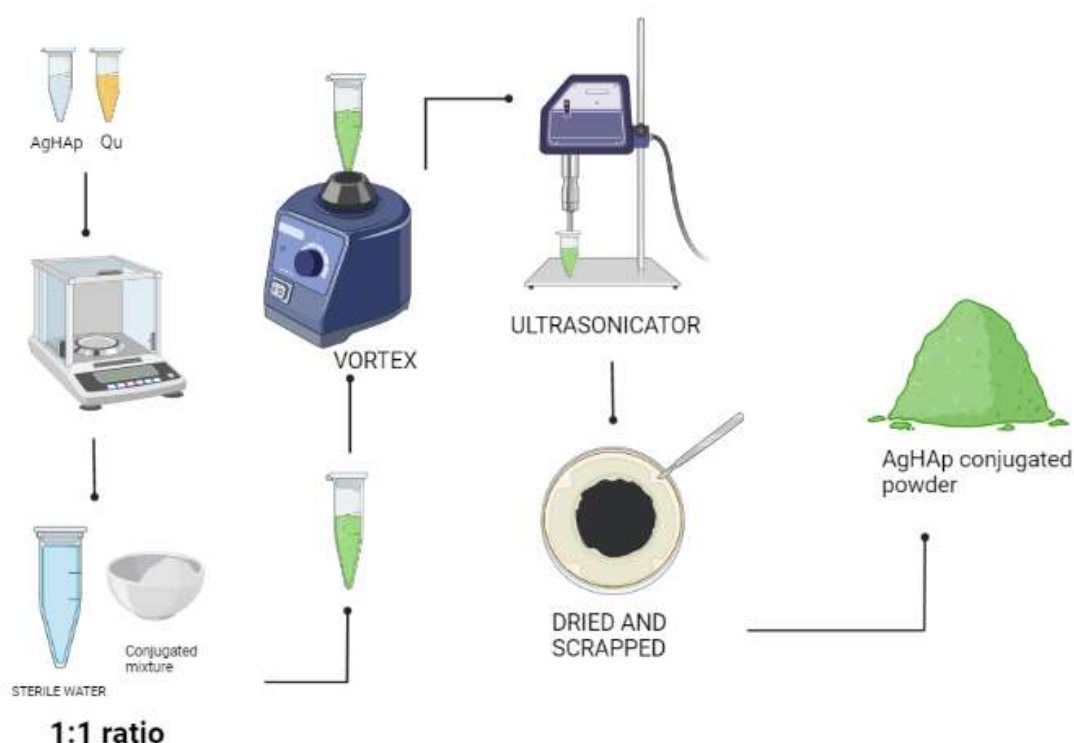


Figure 4.1 Schematic representation of conjugation of AgHAp and Qu

4.2.3 CHARACTERIZATION OF THE SYNTHESIZED AgHAp AND AgHAp-Qu CONJUGATE.

4.2.3.1 FIELD EMISSION SCANNING ELECTRON MICROSCOPY ENERGY DISPERSIVE SPECTROSCOPY

Field emission scanning electron microscopy (FESEM Carl Zeiss, SUPRA® 55) was utilized to investigate the surface morphology of the samples AgHAp and AgHAp-

Qu conjugate. Each sample were vaccum dried and coated with a conductive layer (400 Å) of sputtered gold in order to make them conductive prior to analysis.

4.2.3.2 X-RAY DIFFRACTION

The phase compositions of AgHAp and AgHAp-Qu conjugate samples were determined by X-ray diffraction (XRD, Mini Flex II) using Cu-K α radiation ($\lambda = 0.15406$ nm) at a scan speed of 5°/min. The phase identification was confirmed by comparing the both diffraction pattern of the samples.

4.2.3.3 FOURIER-TRANSFORM INFRARED SPECTROSCOPY

Fourier-transform infrared (FTIR) spectroscopy was performed on the powder samples AgHAp and AgHAp-Qu conjugate using ALPHA FTIR Spectrometer, Bruker. The FTIR spectrum of each sample was scanned over the range from 400 to 4000 cm⁻¹ at spectral resolution of 4 cm⁻¹.

4.2.3.4 UV-VISIBLE SPECTROSCOPY

A UV-visible spectrometer (Epoch Biotek) was used to record the absorbance profile of the synthesized AgHAp and AgHAp-Qu.

4.3 CELL CULTURE

Vero cells and HCT Human colorectal cancer cells were obtained from National Centre for Cell Science (NCCS), Pune and maintained in DMEM medium supplemented with 5% FBS containing 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were grown in T25 flasks at 37°C in a humidified atmosphere containing 5% CO₂. The cells were split every 2-3 days to maintain exponential growth.

4.3.1 CYTOTOXICITY OF AgHAp and AgHAp-Qu CONJUGATE.

Vero cells, MCF7 and HCT Human colorectal cancer cells were seeded at 1×10^4 cells/ well in separate 96 well plates and incubated for 24h at 37°C in 5% CO₂ atmosphere. After the incubation, the medium was discarded and the cells were exposed to various concentrations of (**250-1500µM**) AgHAp and AgHAp-Qu for Vero Cells and (**200-1000µM**) AgHAp and AgHAp-Qu for HCT and MCF7 cells, all these concentration were prepared by dissolving in DMEM media and incubated for 24hr in CO₂ incubator. Following 24hr incubation, MTT (5 mg/mL of stock in PBS) was

added (10 µL/well), and the plates was incubated for 4 hr. After incubating for the required amount of time, 100µL of DMSO was added to each well and incubated for 20 min at room temperature, and the absorbance was read at 570 nm using a multiwell microplate reader .

4.4. DPPH ASSAY

The effect of antioxidants on DPPH is thought to be due to their hydrogen-donating ability. Radical scavenging activities are very important to prevent the deleterious role of free radicals in different diseases, including cancer. DPPH free radical scavenging is an accepted mechanism for screening the antioxidant activity of microbes extract. This method has been used extensively to predict antioxidant activities because of the relatively short time required for analysis.

- A solution of 0.1 mM DPPH (α-diphenyl-β-picrylhydrazyl) is dissolved in 10 ml distilled water and vortex.
- The DHHP solution is added to 100 µl in the 96-well cell culture plate. To added (**200-1000µM**) AgHAp and AgHAp-Qu in the different concentrations to the solution.
- The cell culture plate is gently mixed with hands. The plate was placed in the incubator for 5 minutes at 37°C.
- The cell culture plate is observed at 520 nm in multiple plate reader equipment.

4.5 EFFECT OF AgHAp-Qu ON ADULT MALE ZEBRAFISH (*DANIO RERIO*)

4.5.1 MAINTENANCE AND SPAWNING OF ZEBRAFISH (*Danio rerio*)

Adult fish were fed live brine prawns once daily and dry prawn flakes twice daily while being kept at a constant temperature of 28 °C with a photoperiod of 14 h of light and 10 h of darkness and a pH 7±0.2. For spawning, a 1:2 ratio of mature acclimatised female and male zebrafish was used. Zebrafish were kept in a plastic mesh enclosure while they spawned in order to prevent cannibalism of the released eggs and were covered in black plastic for 12 hours to create a dark environment. After dark incubation, the eggs were exposed to light, and spawning began as soon as the light was switched on and finished in 30 minutes (Dulay et al., 2012). Embryos at the segmentation phase were retrieved, cleaned, and put in a watch glass

following fertilisation. The homogeneity of the embryos was then checked by utilising a stereomicroscope for observation. Prior to its usage in additional tests, the unfertilized or necrotic embryos were removed (Levin et al., 2004).

4.6 ZEBRAFISH EMBRYOTOXICITY AND TERATOGENICITY ASSAY:

Despite potent anticancer activity of AgHAp-Qu, it is equally important to study its toxic nature. Embryotoxicity and teratogenicity is an in vivo toxicity test using zebrafish embryos to evaluate the toxic nature of the AgHAp-Qu. Ten embryos at segmentation phase were transferred into each well containing three different concentrations of AgHAp and AgHAp-Qu (1 mM, 2 mM and 3 mM) and control (embryo water E3 medium containing 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄, 10- 5 % Methylene Blue) placed into each well of the 12-well ELISA plate. The plate was incubated at 26°C ± 1°C. The teratogenic activity was examined using a stereomicroscope after 0, 24 and 48hr of incubation. Morphological endpoint examination of zebrafish was observed following the parameters explained by Nagel (Nagel, 2002).

4.7 HISTOLOGY

Two wild-type female zebrafish of control and AgHAp-Qu treated and aged 3 weeks, respectively. The fish were euthanized with an overdose of tricaine methanesulfonate (MS-222). For microscopic examination, whole fishes were collected, fixed in a 10% neutral buffered formalin (NBF), and dehydrated by hand through an ascending series of graded ethanol solutions to minimize artifacts. The specimens were treated by modifying the standard procedure of hydration/dehydration in order to adapt them to manipulate very small pieces of tissue. In the next step, the tissues were cleared in xylene and embedded in a block of paraffin. The samples were then sectioned at 5 µm using a rotary, floated in a 37 °C water bath, and quickly mounted on glass. The slides were put in an incubation oven at 37 °C for 24 h, and then they were stained manually with Hematoxylin and Eosin (H&E). After staining, the sections were protected by mounting a coverslip over the tissue using a mounting medium to adhere the coverslip to the slide, and they were also examined under a *leica* light microscope.

CHAPTER-5

RESULTS AND DISCUSSION

5.1 VISUAL OBSERVATION

The white HAp powder undergoes color change after chemical reduction and calcination process, which turned into light gray/grayish brown by Ag salt used. Figure 5.1 shows the photographic images of prepared HAp/AgNPs nanocomposite, Quercetin and the conjugate sample AgHAp. This distinctive color change is due to the excitation of surface plasmon resonance that indicates the formation of AgHAp on the powder sample (A), B image shows the color of the bright yellow which is the sample of quercetin. Whereas, C represents the conjugated sample of AgHAp-Qu where the colour is changed into the greenish yellow which shows the conjugation process between the AgHAp and Qu. All the samples were dissolved with 1ml sterile distilled water and were used for the experiments.

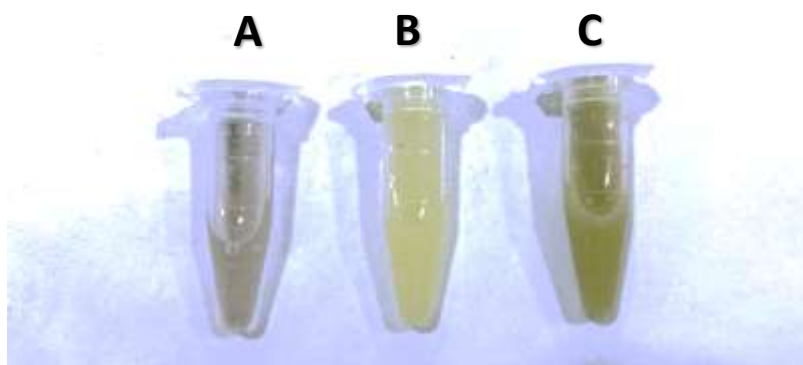


Figure-5.1 Shows the visual observation of A.AgHAp, B.Qu and C.AgHAp-Qu

5.2 RESULTS OF THE CHARACTERIZATION OF THE SYNTHESIZED AgHAp AND AgHAp-Qu CONJUGATE.

5.2.1 FESEM ANALYSIS

Figure Fig.5.2 shows the surface morphologies and results of the elemental composition of AgHAp(A) and AgHAp(B) nanocomposite powder samples. Both

the AgHAp and the conjugated nanocomposite samples had an uneven grain-like shape with an interconnected porous network structure. Regarding the conjugated nanocomposite samples, it is noticeable that some spherical AgNPs(A) have been added to the rod shaped Quercetin(B) particles' surface. This confirm the conjugation between the AgHAp and Qu is successful.

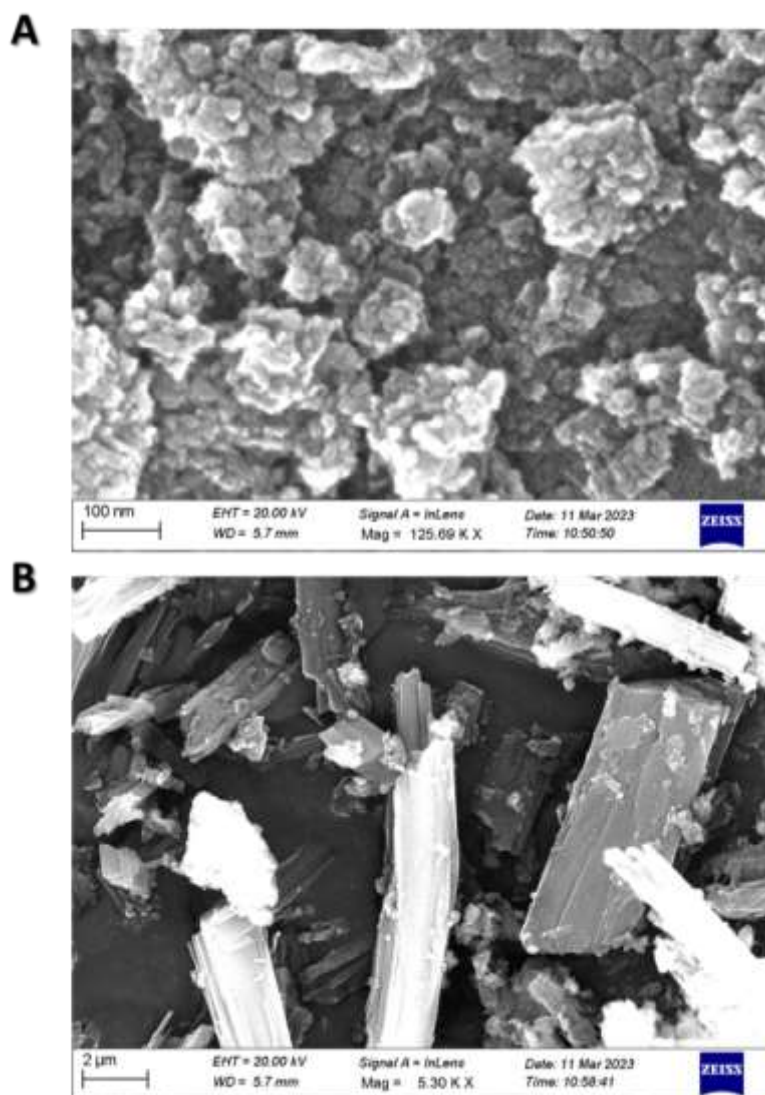


Figure 5.2- FESEM micrographs of (A) AgHAp (99.4nm) and (B) AgHAp-Qu (104.8nm) conjugated nanocomposite powder.

5.2.2 X-RAY DIFFRACTION

Fig 5.3 shows the XRD patterns of the AgHAp(A) and the AgHAp-Qu(B) conjugate and the peaks were identified, indexed by comparing the XRD pattern with both the samples AgHAp and the AgHAp-Qu conjugate. As can be observed in fig, the diffractogram of AgHAp(A) shows the different peaks at the 2θ values 1.72° , 26.02° , 32.35° , 39.86° , 46.87° , 49.61° , 53.46° and 64.18° that are assigned to the (0 0 2), (2 1 0), (2 1 1), (1 1 2), (3 0 0), (2 0 2), (3 1 0), (2 2 2), (3 1 2), (2 1 3), (3 2 1), (4 1 0), and (0 0 4) lattice planes of HAp, respectively. For AgHAp-Qu(B) conjugate nanocomposite sample, additional peaks at 10.82° , 12.47° , 14.24° , 16.18° , 17.33° , 22.28° , 23.90° , 26.64° , 27.43° , 28.67° , 34.03° , 38.65° , 40.36° , 42.01° , 43.89° , 52.20° , 77.38° and 87.66° that are corresponded to the (1 1 1) and (2 0 0) lattice plane of face-centered cubic (fcc) metallic Quare also present along with other characteristic peaks of AgHAp. These results are in accordance with previous reports by Rajendran et al. which demonstrate the successful formation of AgHAp and AgHAp-Qu nanocomposites. Furthermore, no other peaks in addition to AgHAp and Qu are observed in all the AgHAp-Qu nanocomposites, indicating that the chemical reduction and calcination processes did not lead to the instability of the sample to form other phase. It is also apparent that the intensity of AgHAp characteristic peaks in the nanocomposite samples was gradually increased with the AgHAp-Qu sample. This dictate that the AgHAp is successfully conjugated with the Quercetin and the Fig 5.3 (B) show that the peaks from Quercetin are also observed along with the AgHAp.

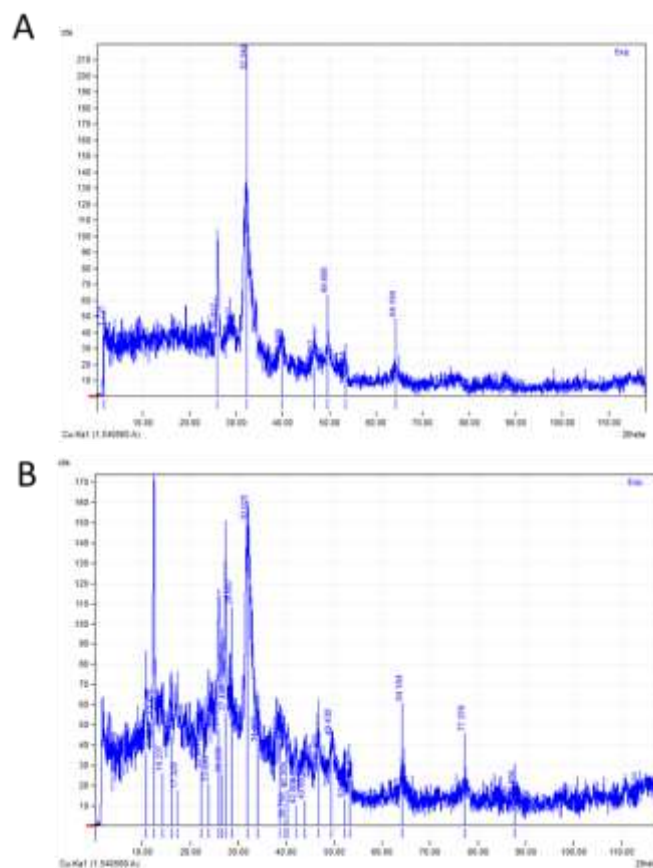


Figure 5.3- XRD patterns of A. AgHAp and B. AgHAp-Qu conjugate nanocomposites.

5.2.3 FOURIER-TRANSFORM INFRARED SPECTROSCOPY

All of the synthesised compounds' functional groups were identified using FTIR spectroscopy.

They have mentioned about the two significant peaks associated with quercetin. The FTIR spectra suggested a significant OH stretch band of at 3400 cm^{-1} due to the presence of H-bonded OH groups. The large presence of OH groups accounts for the solubility of the polymer in the ethanol/water solvent system. Another prominent peak is observed in the 1550–1650 cm^{-1} regions due to the quinone molecule stretching (Milanezi et al., 2019) in his report suggested the presence of significant functional groups like at 3248 cm^{-1} (O=H stretching), 1670 cm^{-1} (C=O stretching), and 1500 cm^{-1} (C-C stretching) which belong to capped QCN based Au

NPs with the most important absorption bands in between lower region of 650 and 1000 cm^{-1} (aromatic compounds).

The strong peaks at 3572 and 633 cm^{-1} in the FTIR spectra of HAp shown in Fig.B correspond to the O-H stretching. The structural hydroxyl group from HAp is stretched and librated. The P-O asymmetric stretching (ν_3), symmetric stretching (ν_1), asymmetric bending (ν_4), and symmetric bending (ν_2) vibrations of the phosphate group, respectively, are attributed to the characteristic bands at 1089-1043, 962, 601-567, and 500-423 cm^{-1} (Kheradmandfard et al., 2020). The sample's adsorbed H_2O 's O-H stretching is what causes the broad peak at 3435 cm^{-1} . The asymmetric stretching and bending vibration modes are linked to the distinctive peaks at 1483-1422 and 880 cm^{-1} , which relate to the CO_3^{2-} 's asymmetric stretching (ν_3) and bending (ν_2) vibration modes., which were found AgHAp's FTIR spectra. The doping of AgNPs onto HAp via chemical reduction and calcination as proposed in contrasts to the Ag-doping method as reported by prior literature (Iqbal et al., 2012), which can result in the development of an extra phase (i.e., -tricalcium phosphate). This study has a benefit in that it prevents the production of further impurity phases. On the other hand, ν_3 PO_4^{3-} 's characteristic band has, which could be explained by a potential interaction between AgNPs and the phosphate group of Hap (B).

Whereas in conjugated AgHAp-Qu, some of the peaks present in both the Quercetin and AgHAp are depicted in the conjugate nanocomposite. The strong peaks at 3572 and 633 cm^{-1} from AgHAp and 1670 cm^{-1} (C=O stretching), and 1500 cm^{-1} (C-C stretching) from Qu and also the stretches from both are observed in the conjugate. This proves that the AgHAp and Qu are conjugated successfully to form the AgHAp-Qu conjugate.

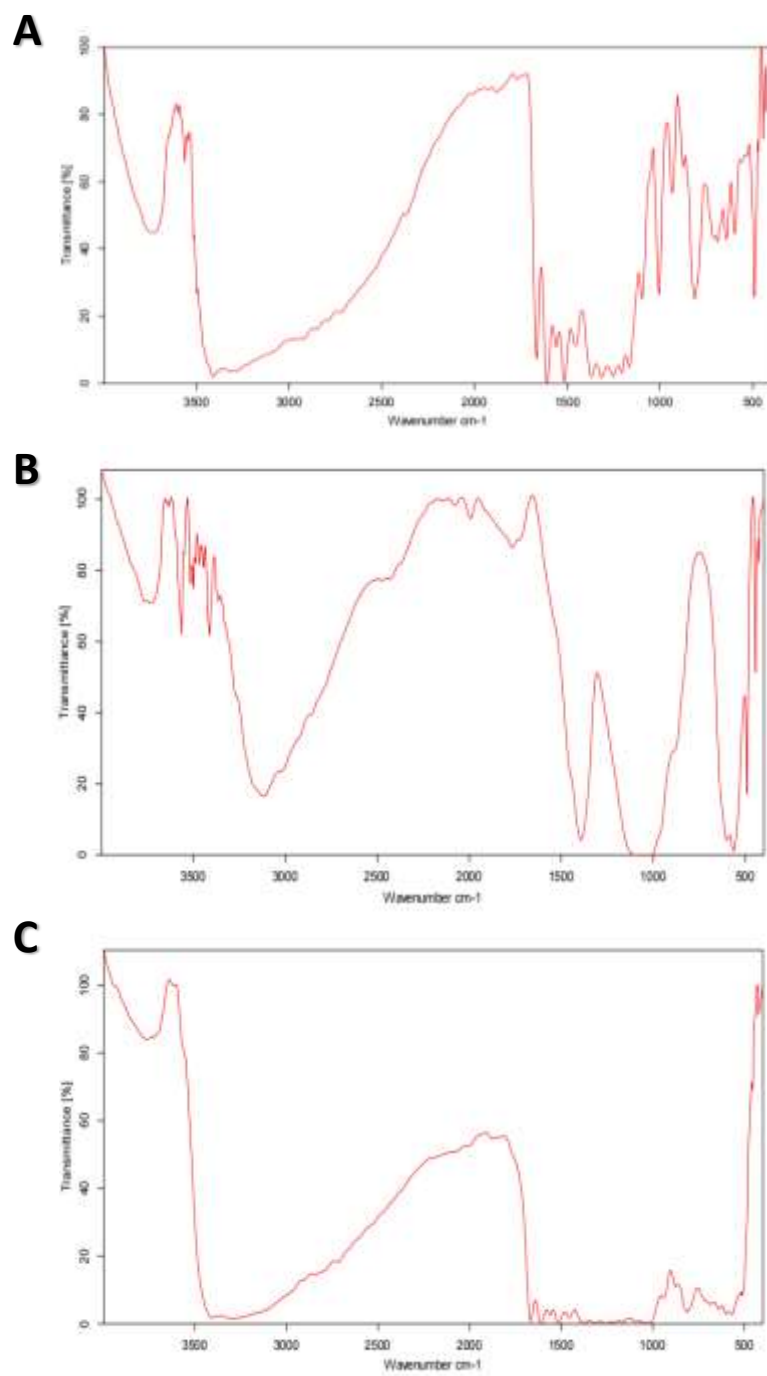


Figure 5.4- FTIR images of A. Qu ; B AgHAp and C. AgHAp-Qu

5.2.4 UV-VISIBLE SPECTROSCOPY

The UV-Vis spectrum of Quercetin (A) has two bands that are attributed to different parts of the conjugated aromatic rings. The first band (Band I) at the 300 - 380 nm corresponds to the cinnamyl and the second band (Band II) at 240 - 280nm corresponds to the benzoyl. The mean maximum OD is at 0.76. The AgHAp(B) has single peak 280-300nm at the 0.365 OD. Whereas, in the conjugate AgHAp-Qu (C) both the peaks from Qu (A) 300 – 380nm, 240 - 280nm; AgHAp (B) 280-300nm and a highest peak 290nm due to the conjugation with Qu on the 0.458 OD were observed in the graph. This confirms the successful conjugation between AgHAp and Qu to form the AgHAp-Qu conjugate nanocomposite.

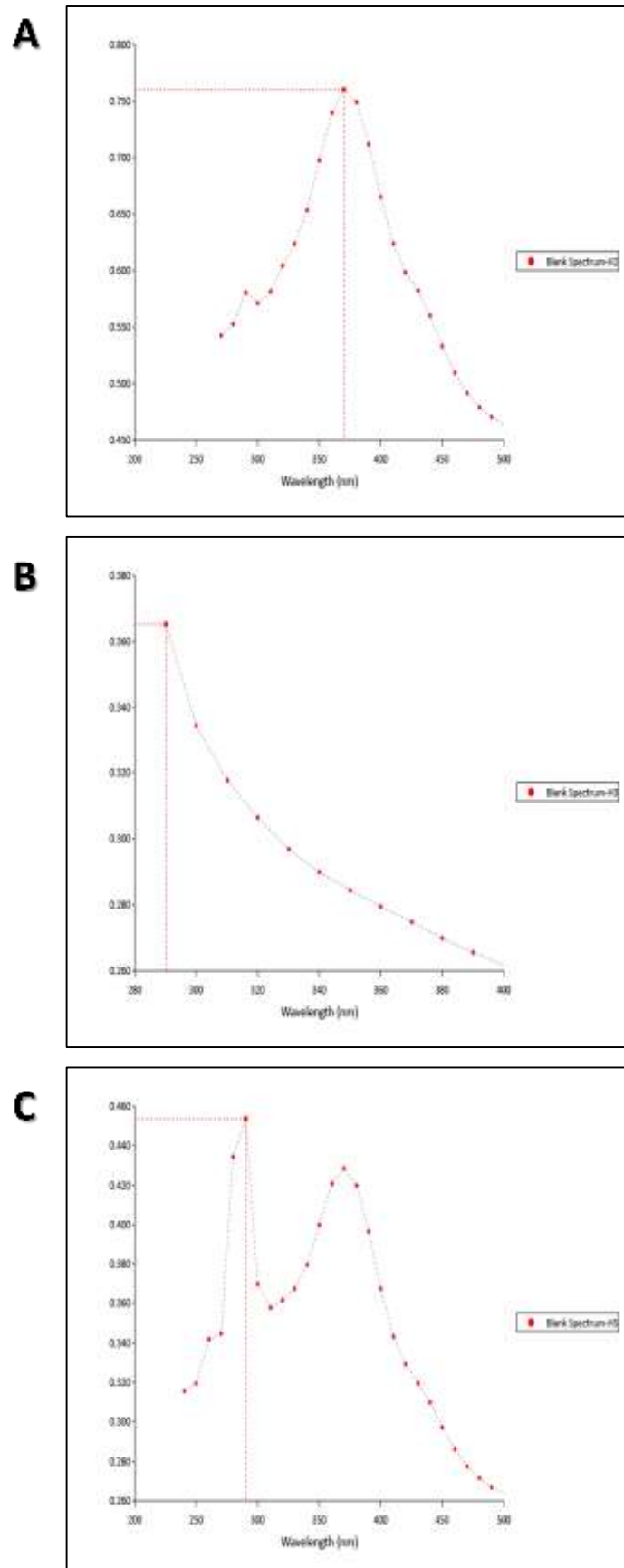


Figure 5.5 - UV spectroscopy images of A. Qu ; B AgHAp and C. AgHAp-Qu

5.3.1 CYTOTOXICITY OF AgHAp and AgHAp-Qu CONJUGATE.

The control, AgHAp and AgHAp-Qu were incubated individually with Vero cells, HCT cells and MCF-7 cells to study the inhibition of the cell growth rate using MTT assay. The corresponding cell viability graph for different concentrations was shown in Fig.5.6-5.8. The absorption percentage of treated cells compared to control cells was used to calculate the percentage of cell viability.

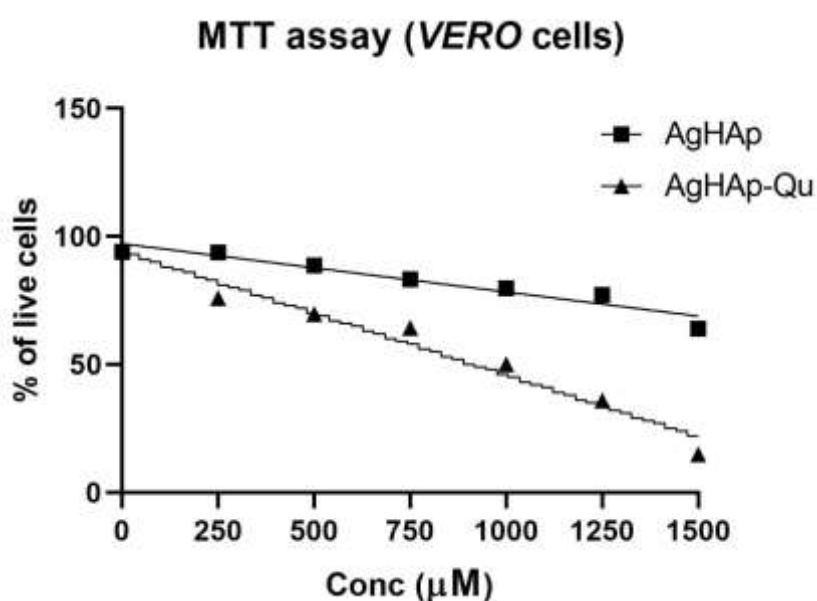


Figure 5.6- MTT assay results of vero cells.

The % of viability were 93.8,88.6,83.2,79.8,77.2,64.4 and 48.5 in 250,500,750,1000,1250 and 1500 μM concentrations for AgHAp whereas for AgHAp-Qu 75.9,69.6,64.3,50.1,35.9 and 14.9 in 250,500,750,1000,1250 and 1500 μM concentrations, IC₅₀-503.1 μM respectively. The % of live cells were decreased along with increasing the concentrations of samples. AgHAp-Qu shown better activity when compared to AgHAp.

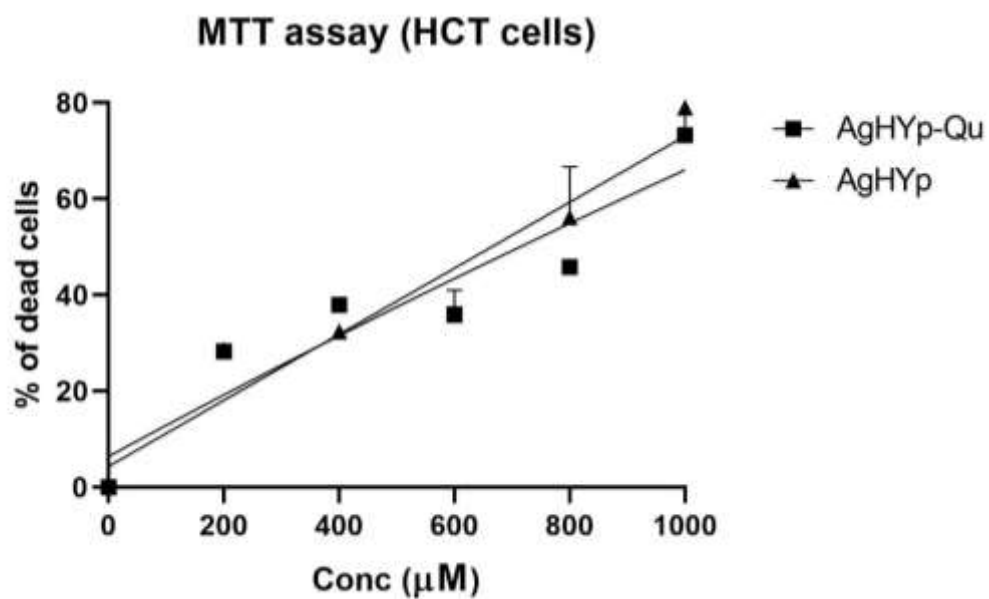


Figure 5.7- MTT assay results of HCT cells.

The % of viability were in 27.6,33.7,34.4,45.5 and 58.9 in 250,500,750 and 1000 μM concentrations for AgHAp whereas for AgHAp-Qu 27.6,36.8,41.4,44.3,67.5 and 14.9 in 250,500,750 and 1000 μM concentrations, IC_{50} -703.6 μM respectively. The % of live cells were decreased along with increasing the concentrations of samples. AgHAp-Qu shown better activity when compared to AgHAp.

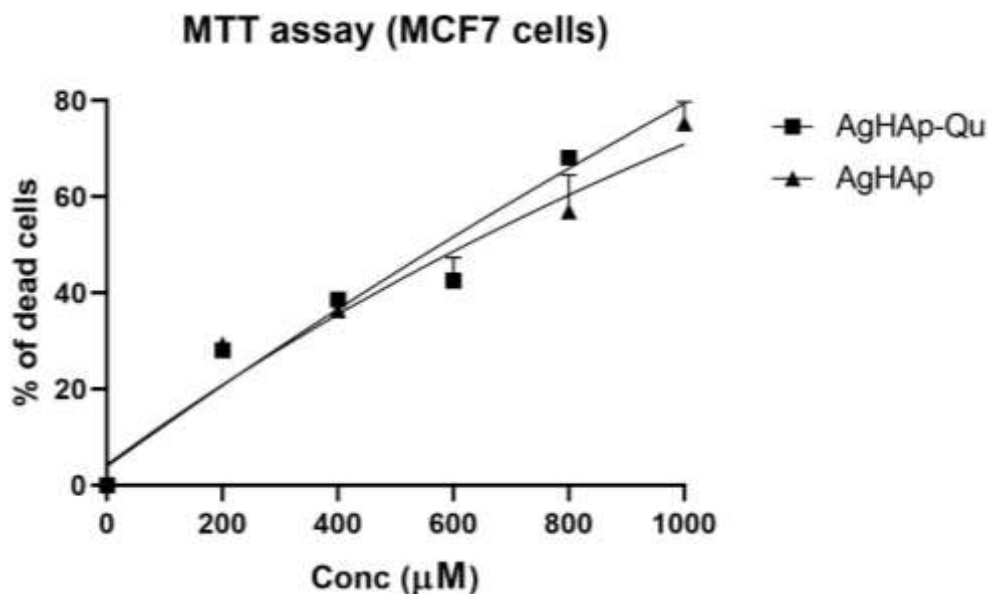


Figure 5.8- MTT assay results of MCF 7 cells.

The % of viability were in 28.4,33.6,37.7,49.3 and 70.9 in 250,500,750 and 1000 µM concentrations for AgHAp whereas for AgHAp-Qu 28.3,38.1,37.9,69.2 and 79.6 in 250,500,750 and 1000 µM concentrations, IC₅₀-824.8 µM respectively. The % of live cells were decreased along with increasing the concentrations of samples. AgHAp-Qu shown better activity when compared to AgHAp.

All these evidences confirms the high therapeutic effect of the drug quercetin which greatly enhances the anticancer activity when conjugated with Silver Hydroxyapatite

5.6 DPPH ASSAY

Free radical scavenging activity of of AgHAp and AgHAp-Qu was estimated using DPPH scavenging activity assay. Different concentrations (200-1000 µM) of AgHAp and AgHAp-Qu were compared for the ability of DPPH radical inhibition. The data showed that the free antioxidants i.e., free radicals of AgHAp and AgHAp-Qu was dose dependent (Figure) and was promoted with increasing concentration. The inhibition capabilities were 16.8,18.5,27.1,41.4 and 48.5 for 200,400,600,800 and 1000 µM (AgHAp) ; 28.7,52.3,59.1,67.2 and 72.4 for 200,400,600,800 and 1000 µM (AgHAp-Qu) concentrations, respectively ,after 12h. As concentration of AgHAp and

AgHAp-Qu increases , the % of radical scavenging activity also increases. Overall, DPPH radical scavenging was higher in Qu conjugated with AgHAp confirming that the antioxidant ability of Qu. Radical scavenging activity was lower in AgHAp but Conjugating it with Qu had increased the radical scavenging activity.

$$\% \text{ Of } INHIBITION = \frac{CONTROL \text{ O.D} - TEST \text{ O.D}}{CONTROL \text{ O.D}} * 100$$

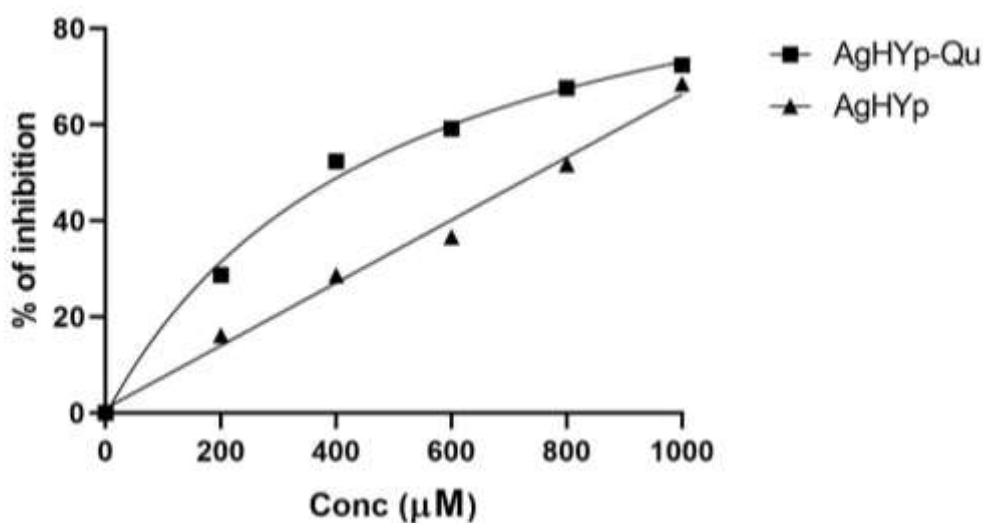


Figure 5.9 - The graph of DPPH radical scavenging activity of AgHAp and AgHAp-Qu

5.7 EMBRYOTOXICITY

Results show that lower concentrations (1-3 mM) of AgHAp and AgHAp-Qu, the zebrafish embryos are healthy and alive. When the concentration increases (3 mM), more deformalities and malformations (delayed hatchability, no development of embryo, rupture of chorion) which can be seen in the increasing concentration of the embryos with the respective time interval. As exposure time interval increases with concentration, the effect of the samples in higher concentration in embryos is also increased. 1-2 mM concentration showed healthy larvae and does not showed

any effect on embryo. Hence, optimum concentration does not show any toxicity effect on zebrafish embryos.

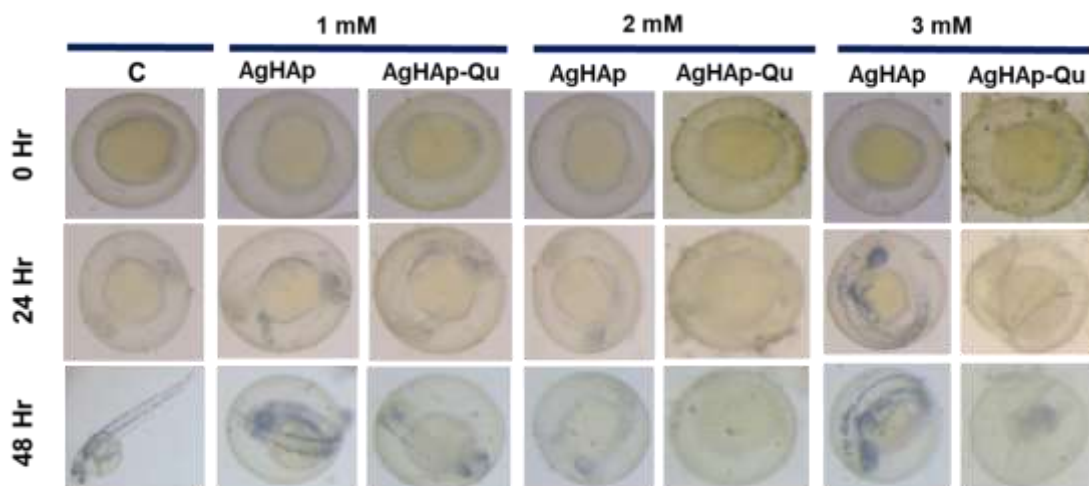


Figure 5.10- The developmental toxic effect of AgHAp and AgHAp-Qu on zebrafish Embryos

5.8 HISTOLOGY

Morphological responses of the gills to AgHAp-Qu exposure were characterized by increased cellularity in the interlamellar space (Fig. 5.11). However, changes in gill filament width following AgHAp-Qu exposure varied significantly. In 15 days, AgHAp-Qu nanoparticle exposure caused increase in gill filament width. AgHAp-Qu exposure both increased gill filament widths by approximately twofold over control values. Gills taken from nanoparticulate AgHAp-Qu -exposed fish were significantly wider than those from control.

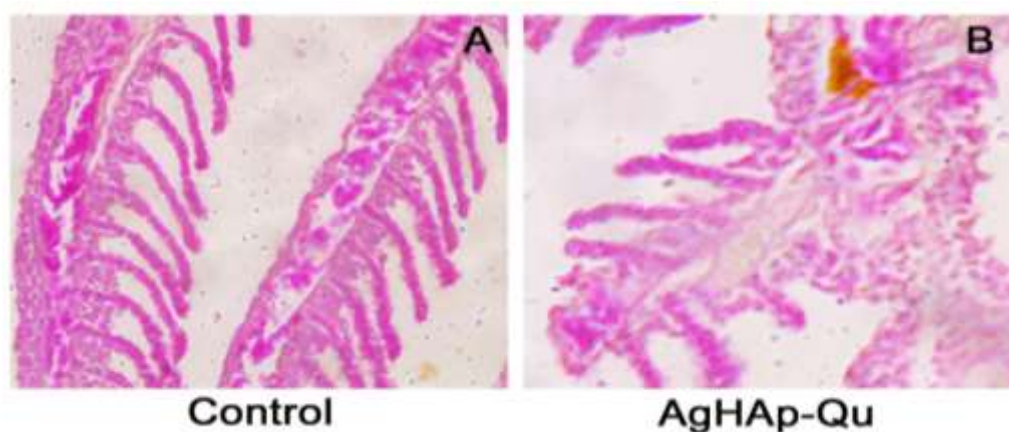


Figure 5.11 Histopathological analysis of adult Zebrafish gills treated with AgHAp-Qu

To further investigate the effects of AgHAp-Qu on liver a histopathological analysis was conducted. Exposure to AgHAp-Qu elicited much hepatocellular atrophy and necrosis, and increased hepatic plate gap in the liver treated with 5mM AgHAp-Qu. Therefore, the damaging effects of AgHAp-Qu on the liver warrant scientific attention since the liver is a pivotal organ in the metabolism and detoxification of xenobiotics.

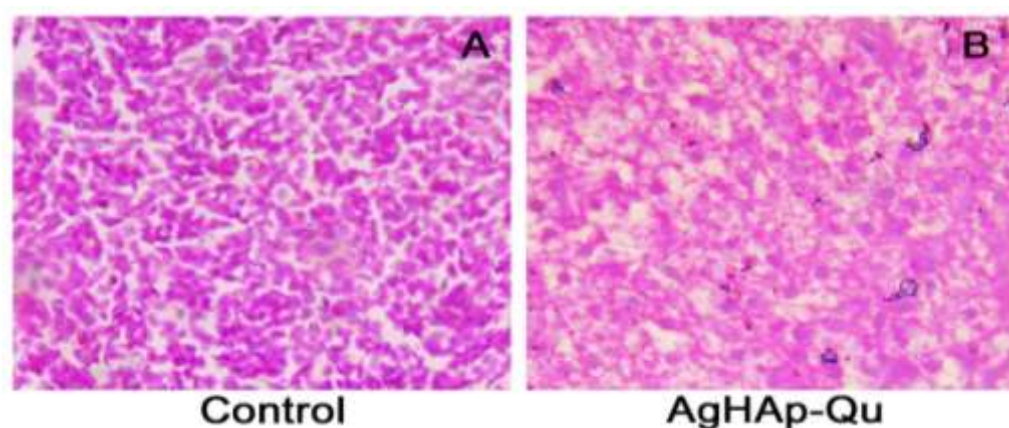


Figure 5.12 -Histopathological analysis of adult Zebrafish liver treated with AgHAp-Qu

5.9 PROPOSED MECHANISM FOR THE FORMATION OF HAp/AgNPs NANOCOMPOSITE.

Fig 5.13 illustrates the hypothesised formation pathway of HAp/AgNPs nanocomposites, which involves the chemical reduction of an Ag salt precursor by

DMF and stabilisation provided by PVA. It is widely known that the DMF may function as both a solvent and a potent reducing agent for the reduction of noble metallic ions. When AgNO_3 is added to DMF, the DMF's $-\text{C}=\text{O}$ group can readily be converted to a $-\text{COOH}$ group in the presence of Ag^+ ions with a strong oxidation potential. This will then cause the reduction of $\text{Ag}(\text{I})$ ions into metallic $\text{Ag}(0)$ metal by contributing electrons during the oxidation process.

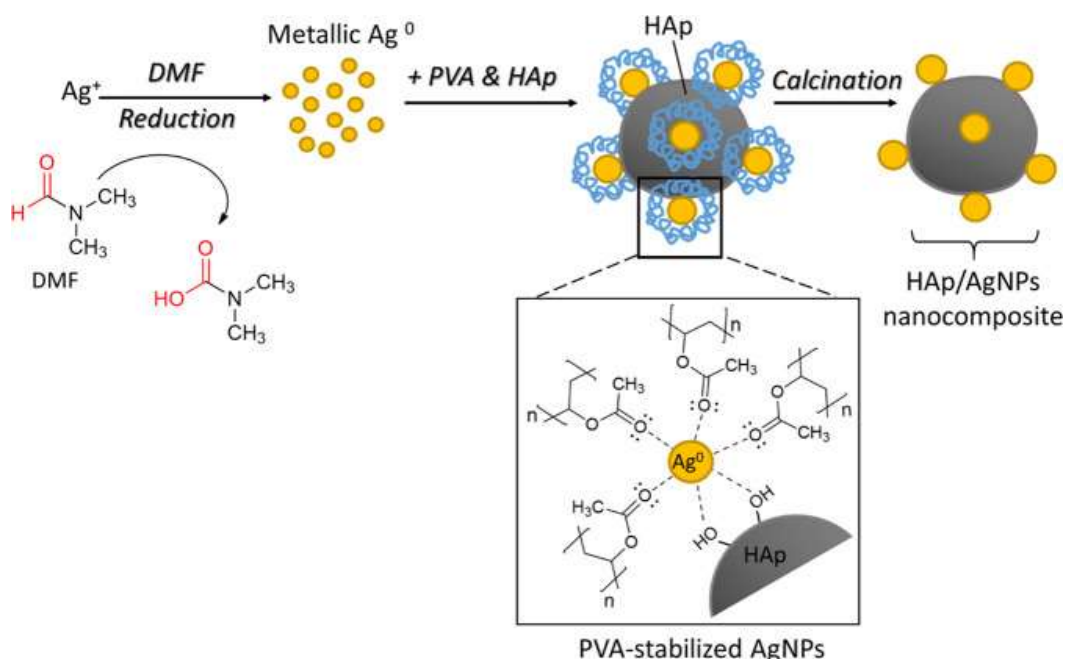


Figure 5.13 - Proposed mechanism for the formation of HAp/AgNPs nanocomposite via chemical reduction route using DMF and PVA as reducing and capping agents, respectively (Bee et al., 2021).

PVA will operate as a capping agent when metallic Ag solution is added to the HAp/PVA suspension, preventing excessive Ag particle agglomeration during the coalescence process. AgNPs are anticipated to be stabilised in this situation by strong interactions between their surfaces and the oxygen atoms in PVA, leading to the formation of a protective coated layer on their surface. By introducing steric repulsive interactions between AgNPs, this layer will prevent the agglomeration of particles. As AgNPs attach to and deposit in the reaction mixture, HAp will also

operate as a nucleating site. This is due to the strong affinity for Ag atoms that the electron-rich -OH groups on the HAp surface exhibit, which allows for Ag atom adsorption and coordination via oxygen atom on the HAp. Thus, after the reaction is complete, PVA can be thermally removed from the HAp/AgNPs nanocomposite sample (Bee *et al.*, 2021).

DISCUSSION

Quercetin has attracted a lot of attention due to its antioxidant (Bao *et al.*, 2017), anti-inflammation anti-apoptotic and anti-cancer properties, meanwhile due to low solubility in water and low bioavailability, cannot be still used as a drug and needs to undergo manipulation (Massi *et al.*, 2017). The manipulation of materials at the nanoscale can modify their bioactivity and main properties such as solubility, circulation period, and access to the target tissue. Furthermore, Nanoparticles are especially useful for delivering water-insoluble compounds because of their tiny size can arise the absorption and bioavailability of the compounds (Wu *et al.*, 2008). HAp NPs' chemical structure provides good opportunities for loading and delivering a wide variety of anticancer medicines in a sustained, extended, and targeted way. Another strategy for delivering advanced anticancer effects, such as the capacity to inhibit the growth and metastasis of cancer cells by activating particular cell signalling pathways, is the incorporation of specific therapeutic elements into the basic composition of HAp NPs. This strategy can be used alone or in combination with drug release by using various surface modification techniques. Also, HAp NPs can readily be transformed into smart anticancer agents that make it easier to target and destroy various type of cancers (Kargozar *et al.*, 2022). HAp NPs can inhibit cancer cell growth, migration, and invasion via downregulating the FAK/PI3K/Akt cell signaling pathway, both in vitro and in vivo was reported by Wang *et al.*, (Wang *et al.*, 2021). Oxidative stress is defined as an imbalance between production of free radicals and reactive metabolites, so-called oxidants or reactive oxygen species (ROS), and their elimination by protective mechanisms, referred to as antioxidants. This imbalance leads to damage of important biomolecules and cells, with potential impact on the whole organism. HAp NPs can also cause oxidative stress-induced apoptosis in cancer cells by lysosomal and mitochondrial-dependent pathways,

including caspase. Bagad and Khan encapsulated Qu into poly (n-butyl cyanoacrylate) NPs, with or without polysorbate-80 coating, for oral administration. They are reported that the coated NPs improved the oral bioavailability of Qu and enhanced the Qu concentration in the brains of rats (Bagad and Khan, 2015). Prior experimental studies have confirmed the suitability of drug-loaded multi-modal HAp NPs as effective substances for tumor metastasis resisting therapy (TMRT) and tumor metastasis targeting therapy (TMTT) (Xiong et al., 2018). Therefore, our results verify the strong conjugation between AgHAp and QU in AgHAp-Qu conjugate.

Antioxidants are chemicals that interact with and neutralize free radicals, thus preventing them from causing damage. Antioxidants are also known as “free radical scavengers.” Numerous studies indicated Qu loaded NPs, had more anti-radical and antioxidant activities as compared to free Qu. Encapsulating of Qu and catechin into poly D, L lactide-co-glycolide NPs promoted its lipid oxidation inhibition and antioxidant capacities. Additionally, loading Qu into Eudragit® E (aminoalkyl methacrylate copolymers) - polyvinyl alcohol NPs increased the anti-radical and antioxidant activities of Qu (Wu et al., 2008). In the current study, the conjugation of Qu with AgHAps strictly reduced the DPPH scavenging and antioxidant of Qu in a non-cellular medium. Researchers reported replacing the hydroxyl groups of Qu with glucose has reduced its antioxidant effects (Graefe et al., 2001). HAp NPs with mesoporous structures (i.e., pore sizes in the range of 2– 50 nm) possess high pore volumes and adequate pore sizes for storing therapeutic molecules and targets as compared with their macroporous (pore sizes > 50 nm) and microporous (pore sizes <2 nm) counterparts (Yasun et al., 2020). Therefore, the conjugation of Qu through the functional groups -responsible for its anti-radical and antioxidant properties- with AgHAps may have been the cause of the reduction in its activities. We predict that the anti-radical and antioxidant activities of QNPs would be improved after passing time due to Qu release from QNPs and the activation of Qu functional groups. Although the DPPH scavenging and antioxidant assays results demonstrated AgHAp-Qu had less antioxidant activities as compared to free AgHAp.

The MTT assay measures cell respiration and the amount of formazan produced is proportional to the number of living cells present in culture. An increase or decrease

in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the drug. In this present study, the obtained results showed that the low doses of AgHAp and AgHAp-Qu does not affect much viability of cancer cells. But, along with increasing concentration and incubation period, their protective effects were reduced, and finally, altered to adverse effects at high concentrations in agreement with previously published data. The MTT assay results in healthy kidney cells (vero cells), the % viability of cells were higher compared to the cancer cell lines. This indicates that the AgHAp-Qu conjugate's cytotoxicity effect is lower in vero cells compared with cancer cells. Echeverry and colleagues indicated that the antitoxic effects of Qu against H₂O₂ on primary cerebellar granule neurons of rats declined with elevating Qu doses (Echeverry et al., 2010). Dajas and colleagues declared Qu at doses $\geq 100 \mu\text{M}$ may have a pro-oxidative activity and proposed the presence of an ortho-dihydroxy structure in the B ring and thus, susceptibility to auto-oxidation of Qu or be converting to pro-oxidant intermediates by enzymatic oxidation could be the causes of the Qu pro-oxidative activity (Dajas et al., 2015). Additionally, Barnes et al declared the interaction between H₂O₂ and Qu can lead to oxidation of Qu and production of toxic radicals from Qu (Barnes and Schug, 2014). Therefore, Qu and QNPs have dose- and time-dependent antioxidant effects and may be cytotoxic in high concentrations and long periods.

Generally, two main approaches are being applied to improve the luminescence intensity of doped HAp nanostructures. An increase in dopant content in the HAp structure is proposed as the first method; however, this may lead to fluorescence quenching and greater cytotoxicity. The second strategy is related to calcination at high temperatures to increase the luminescence intensity by improving the HAp particle crystallinity as well as enhancing the diffusion of dopants into the HAp crystalline lattice. However, calcination may increase the size and agglomeration of HAp crystals, interfering with cellular uptake. As an alternative approach, co-doping of HAp NPs has been investigated to improve luminescence intensity (Kargozar et al., 2022).

X-ray powder diffraction (XRD) is a rapid analytical technique primarily used for phase identification of a crystalline material and can provide information on unit cell

dimensions. The analyzed material is finely ground, homogenized, and average bulk composition is determined. X-ray diffraction is based on constructive interference of monochromatic X-rays and a crystalline sample. These X-rays are generated by a cathode ray tube, filtered to produce monochromatic radiation, collimated to concentrate, and directed toward the sample. FTIR stands for “Fourier transform infrared” and it is the most common form of infrared spectroscopy. All infrared spectroscopies act on the principle that when infrared (IR) radiation passes through a sample, some of the radiation is absorbed. The radiation that passes through the sample is recorded. UV-Vis spectroscopy is an analytical technique that measures the amount of discrete wavelengths of UV or visible light that are absorbed by or transmitted through a sample in comparison to a reference or blank sample. The Scanning electron microscope works on the principle of applying kinetic energy to produce signals on the interaction of the electrons. These electrons are secondary electrons, backscattered electrons, and diffracted backscattered electrons which are used to view crystallized elements and photons. Based on XRD, FESEM-EDX, and HRTEM results, the amount and size of AgNPs decorated on the surface of HAp particles are depended on the concentration of AgNO_3 used. It was revealed that increasing AgNO_3 concentration will increase the amount and size of the decorated AgNPs (Bee et al., 2021). The synthesized ZnONP and ZnO-NP-DP were characterized using standard characterization tools such as XRD, FESEM and FTIR spectroscopy. The dipeptide conjugated with zinc oxide nanoparticles showed potent cytotoxic activity against liver cancer cell HepG2 through inducing cell cycle arrest in our previous study (Gayathri et al., 2021). Likewise, in this study the size of the AgHAp and AgHAp-Qu were characterized using SEM, FTIR, XRD and UV-spectroscopy and the results helped that conjugation was successful between the samples of AgHAp and AgHAp-Qu conjugate.

In addition to this, the toxicity study on zebrafish embryos concluded that ZnONP-DP was toxic and produced teratogenic activities against zebrafish embryo only at higher concentrations. These significant results strongly impose that dipeptide conjugated with ZnO nanoparticles could be a valuable resource of bioactive compounds that can be used in the treatment against aggressive cancer cells (Wang et al., 2022). Oxidative stress, neuroinflammation and apoptosis in the brains were examined to learn more about mechanisms of action related to quercetin. The

results showed that quercetin at the lower concentrations exerted beneficial effects on shoaling and anxiety behaviors. On the contrary, when quercetin was up to 1000 µg/L, it exerted detrimental effects shown as decreases of movement and increases of anxiety behaviors (Wang et al., 2022). Likewise in this study, as the concentration of AgHAp and AgHAp-Qu increases more deformalities and malformations were observed in the zebrafish embryos. Whereas in lowest concentrations, the embryos were healthy without any malformations. This says that the higher concentration gives toxicity effect and lower concentration does not harm any.

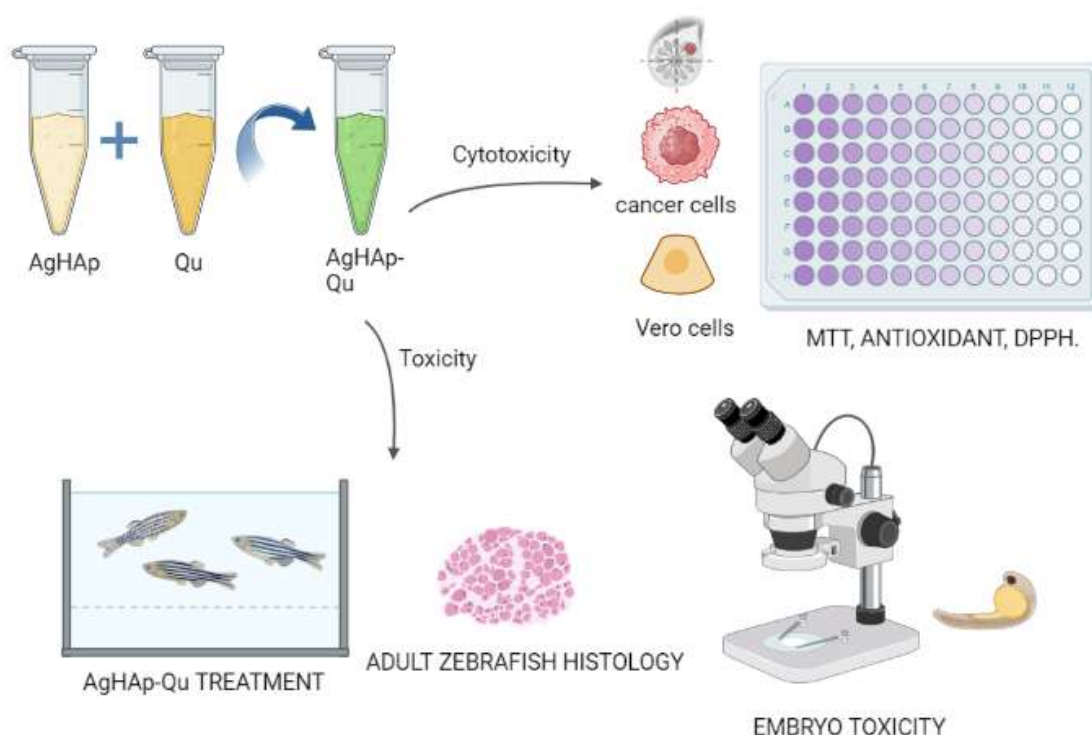


Figure 5.14 Schematic representation of the theme of this present study. AgHAp – Silver Hydroxyapatite; Qu- Quercetin; AgHAp-Qu – Quercetin loaded Silver Hydroxyapatite; MTT - 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DPPH - 2,2-diphenyl-1-picrylhydrazyl.

CONCLUSION

In finalisation, it was successful to create AgHAp nanoparticles and conjugate them with Quercetin. Using various characterisation techniques such as FESEM, XRD, FTIR, and UV-spectroscopy, the synthesised AgHAp and AgHAp-Qu conjugate were examined. Qu has antioxidant, anti-inflammatory, anti-carcinogenic, antiviral, and antibacterial activity, that may increase disease resistance and AgHAp enhancing anticancer properties and mostly in drug delivery. To increase its anticancer effect in the current work Qu was conjugated with the AgHAp nanoparticle. The antiproliferative study's findings unambiguously show that AgHAp-Qu therapy eliminates cancer cells more effectively than AgHAp treatment alone. Additionally, it was shown that AgHAp-Qu increased the amount of apoptosis. In comparison to AgHAp nanoparticles alone, Silver hydroxyapatite conjugated with Quercetin significantly inhibited the growth of cancer cells. Furthermore, by concentrating on the molecular mechanism of starting apoptosis through the signalling pathways of AgHAp-Qu for developing nanoparticle-Qu based anticancer agents against cancer, AgHAp conjugated with quercetin could be a new class of nano-drug for treating cancer. Additionally, the toxicity investigation on zebrafish embryos found that AgHAp-Qu was toxic and only exhibited teratogenic effects on zebrafish embryos at higher concentrations. These important findings strongly suggest that Quercetin coupled with AgHAp nanoparticles may be an important source of bioactive compounds that can be employed to treat cancer cells that are invasive.

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