ELUCIDATING THE PHYSICOCHEMICAL CHARACTERISTIC AND CYTOTOXIC ACTIVITY OF CHITOSAN BASED NANOPARTICLES WITH *Gynura Procumbens* EXTRACT AS A NEW MODULAR TREATMENT AGAINST CANINE MAMMARY GLAND TUMOR

MUHAMMAD AL-SYAFIQ BIN ABDUL HALIM

DOCTOR OF VETERINARY MEDICINE

2023

Elucidating the Physicochemical Characteristic and Cytotoxic Activity of Chitosan Based Nanoparticles with *Gynura Procumbens* Extract as A New Modular Treatment Against Canine Mammary Gland Tumor

By

Muhammad Al-Syafiq Bin Abdul Halim
(D19A0029)

A Research Project Submitted to The Universiti Malaysia Kelantan in partial fulfilment of the requirements for the degree of Doctor of Veterinary Medicine

Faculty of Veterinary Medicine
UNIVERSITI MALAYSIA KELANTAN

2023

ORIGINAL LITERARY WORK DECLARATION

I hereby certify that the work embodied in this thesis is the result of the original research and has not been submitted for a higher degree to any other University or Institution.

☐ **OPEN ACCESS** I agree that my thesis is to be made immediately available as hardcopy or online open access (full text).

☐ **EMBARGOES** I agree that my thesis is to be made available as hardcopy or online (full text) for a period approved by the Post Graduate Committee.
Dated from ___until _____.

☐ **CONFIDENTAL** (Contains confidential information under the Official Secret Act 1972) *

☐ **RESTRICTED** (Contains restricted information as specified by the organisation where research was done) *

I acknowledge that Universiti Malaysia Kelantan reserves the right as follows.

1. The thesis is the property of Universiti Malaysia Kelantan
2. The library of Universiti Malaysia Kelantan has the right to make copies for the purpose of research only.
3. The library has the right to make copies of the thesis for academic exchange.

_____ _____

SIGNATURE OF CANDIDATE SIGNATURE OF SUPERVISOR

_____ _____

NRIC/PASSPORT NO. NAME OF SUPERVISOR
DATE: DATE:

CERTIFICATION

This is to certify that we have read this research paper entitled 'Elucidating The Physicochemical Characterisations And Cytotoxic Activity Of Chitosan Based Nanoparticles Functionalized With *Gynura Procumbens* Extract As A New Modular Treatment Against Canine Mammary Gland Tumor by Muhammad Al-Syafiq Bin Abdul Halim, and in our opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course DVT55204 – Research Project.

_____

Ts. Dr. Muhammad Luqman Bin Nordin
DVM (UMK), MVSc (UPM),
PhD (UKM)
Senior Lecturer
Faculty of Veterinary Medicine
Universiti Malaysia Kelantan
(Supervisor)

_____

Associate Professor Dr. Rumaizi Bin Shaari
DVM (UPM), MVM (UPM) & PhD (University of Adelaide)
Associate Professor
Faculty of Veterinary Medicine
Universiti Malaysia Kelantan
(Co-supervisor)

ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial requirement on the course DVT55204 – Research Project.

Abstract: Canine mammary gland tumours (CMGT) are common in intact bitches. Although surgical excision and chemotherapy are the main therapies for CMGT, but these approaches frequently result in high recurrence rates and adverse effects, including cardiotoxicity and nausea. Chitosan, a natural polysaccharide found in crustaceans, and (GP), a plant that has been traditionally used to treat a variety of ailments, have been shown to have potential as part of cancer treatment with fewer side effects. However, their used in veterinary medicine, particularly in treating CMGT is still unestablished. Therefore, this study aims to analyse the physicochemical characteristics of chitosan nanoparticle formulation, assess in vitro cytotoxic effects of chitosan nanoparticle and GP extract on mammary tumor cells, and identify phytochemical constituents in GP extract. Investigations encompassed physicochemical analysis of the chitosan formulation using dynamic light scattering (DLS) zetasizer, evaluation of in vitro cytotoxic effects using 4T1 cell lines, and identification of phytochemical constituents in GP extract qualitative and quantitatively. The cytotoxic activity of Chitosan, Chitosan-GP and GP extract was assessed using MTT assay against 4T1 cell line for 72 hours. The cell scratch migration assay was performed to investigate the migration inhibition potential of these substance. The result demonstrated Chitosan nanoparticles, produced using the ionic gelation technique, showed a size of 340 nm, a polydispersity index of 0.3, and zeta potential of 14 mV. The GP extract revealed the presence of alkaloids and tannins in GP extract, with significant total phenolic content (79.808 mg of GAE/g) and total flavonoid content (61.621 mg of QE/g), respectively. Cytotoxicity assessments demonstrated highly active cytotoxic activity of Chitosan and chitosan-GP extract against 4T1 cells. However, GP extract was shown weak cytotoxic against 4T1 cells. The Cell Migration Assay revealed inhibitory effects with treatment of Chitosan and Chitosan -GP extract shown the most significant inhibition followed by the GP extract. Results from this experiment indicate that GP extract, Chitosan, and Chitosan-GP exhibit cytotoxic activity against 4T1 cells, suggesting a potential modular treatment for CMGT. Overall these findings would create potential of new paradigm in the search of an effective therapeutic strategies against CMGT in veterinary oncology.

Keywords: Canine mammary gland tumour (CMGT), Chitosan nanoparticles, Physicochemical characteristics, Cytotoxic activity, *Gynura procumbens extract*

ABSTRAK

Abstrak: Tumor payu dara anjing umumnya berlaku pada anjing betina yang masih utuh, dan walaupun pembedahan dan kemoterapi adalah rawatan utama, ia sering menyebabkan kadar kekambuhan yang tinggi dan kesan sampingan yang tidak diingini, seperti kardiotoksikiti dan mual. Kitosan, sejenis polisakarida semulajadi yang terdapat dalam krustasia, dan Gynura procumbens (GP), sejenis tumbuhan yang digunakan secara tradisional untuk pelbagai masalah kesihatan, menunjukkan potensi dalam terapi kanser dengan kesan sampingan yang kurang. Walau bagaimanapun, penggunaan mereka dalam perubatan veterinar, terutama dalam rawatan kanser payu dara anjing, masih tidak terbukti. Kajian ini bertujuan untuk menganalisis ciri-ciri fizikokimia formulasi nanopartikel kitosan, menilai kesan sitotoksik in vitro nanopartikel kitosan dan ekstrak GP terhadap sel tumor payu dara, dan mengenal pasti konstituen fitokimia dalam ekstrak GP. Penyiasatan melibatkan analisis fizikokimia formulasi kitosan menggunakan teknik penyerakan gel ionik dengan pengukuran saiz menggunakan zetasizer, penilaian kesan sitotoksik in vitro menggunakan sel 4T1, dan pengenalpastian konstituen fitokimia dalam ekstrak GP dengan ujian kualitatif dan kuantitatif. Akhirnya, aktiviti sitotoksik diukur dengan merawat sel 4T1 selama 72 jam dengan Kitosan, Kitosan GP, dan ekstrak GP secara individu dan menggunakan ujian MTT untuk mengukur peratusan perencatan sel, serta ujian pergerakan calar sel untuk mengkaji potensi perencatan pergerakan bahan ini. Dari eksperimen kami, nanopartikel kitosan yang dihasilkan menggunakan teknik penyerakan gel ionik menunjukkan saiz 340 nm, indeks polidespersiti 0.3, dan potensi zeta 14 mV. Ekstrak GP menunjukkan kehadiran alkaloid dan tanin, dengan kandungan fenolik total yang signifikan (79.808 mg GAE/g) dan kandungan flavonoid total (61.621 mg QE/g). Penilaian sitotoksik menunjukkan aktiviti sitotoksik yang tinggi untuk Kitosan & kitosan GP terhadap sel 4T1 sementara aktiviti sitotoksik yang lemah bagi Gynura procumbens diperhatikan apabila dirawat secara individu. Ujian Pergerakan Sel mendedahkan kesan perencatan. Keputusan dari eksperimen ini menunjukkan bahawa ekstrak GP, Kitosan, dan Kitosan-GP menunjukkan aktiviti sitotoksik terhadap sel 4T1, mencadangkan potensi rawatan modular untuk CMGT. Temuan ini menekankan kepentingan strategi terapeutik inovatif dalam onkologi veterinar.

Kata Kunci: Tumor Kelenjar Susu Anjing (TKSA), Nanopartikel Kitosan, Ciri-ciri Fizikokimia, Aktiviti Sitotoksik, , Ekstrak *Gynura procumbens*

ACKNOWLEDGEMENT

In the name of Allah, the Most Gracious and Most Merciful. An utmost gratitude goes to Allah, with His blessing and strength, I can finally complete my Final Year Research Project. I dedicate my dissertation work to my beloved family and person. My deepest gratitude to my father Abdul Halim bin Mohamad, my late mother Fauziah Binti Awang @Abdullah, my siblings and close friends. I am extremely grateful for their valuable sacrifices, thoughts, and prayers for my future

My sincerest gratitude and appreciation to my supervisor, Dr. Muhammad Luqman bin Nordin for thorough supervision, patience, critics, knowledge and guidance throughout the process of completing this project. It was a great privilege and honour to work and study under his guidance.

My profound appreciation to Dr Abdin and Dr Mariam, lecturers from faculty of pharmacy UNIKL Royal College Medicine Perak, for sharing their knowledge, information and commitment all though my project, I also appreciate the UMK laboratory staffs especially Ms Nani Izreen that has been important part of my project by guiding and providing the information and practical skill needed in order to complete my project

Last but not least, my special thanks to friends, and class of DVM 2019/2024 UMK for giving me never ending support, encouragement, motivation and prayers until the end of the project.

TABLE OF CONTENT

\

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABBREVIATIONS	DEFINITION
UMK	Universiti Malaysia Kelantan
UPM	University Putra Malaysia
DVM	Doctor of Veterinary Medicine. *Refers to undergraduates' veterinary medicine students*
FPV	Faculty of Veterinary Medicine
UNIKL	University Kuala Lumpur
CMGT	Canine Mammary Gland Tumor
FCR	Folin-Ciocalteu reagent
TPP	Sodium Tripolyphosphate
GP	*Granura Procumbens*
CNp	Chitosan Nanoparticles
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
CGM	Complete Growth Medium
TPC	Total Phenolic Content
TFC	Total Flavonoid content
GAE	Gallic Acid Equivalent
QE	Quercetin Equivalent

LIST OF SYMBOLS

SYMBOLS	DEFINITION
>	More Than
<	Less Than
%	Percentage
g	Gram
mg	Milligram
C	Celsius
nm	Nanometre
mV	Millivolt
µg/ml	Microgram Per Millilitre
µL	Microlitre

1.0 INTRODUCTION

Dogs are very faithful, clever, and loyal domestic pet animals. These days many households keep dogs as pets and often consider them to be human's best friends, they live and travel together, which means that they are exposed to carcinogens and outside risk factors. Typical life expectancy for dogs is approximately 11 to 14 years old based on respective breeds (Montoya et al., 2023). Older dogs (>10 years) are more likely at high risk of health issues including cancer (Kim et al., 2018). Canine mammary gland tumor (CMGT) highest prevalence is in intact bitch according to Goldschmidt et al. (2011), Vascellari et al. (2016) found that 250 out of every 100,000 dogs had CMGT diagnoses, or 56% of all canine neoplasm cases. According to Baba and Catoi (2007), the prevalence is more common in several breeds, including, English Setter Spaniels, English Spaniels, Poodles, and German Shepherds, and lower in Boxer types and Chihuahuas. Age and hormonal instability (estradiol-17β, oestrogen and prolactin) are other factors that are associated with CMGT incidence (Spoerri et al., 2015). CMGT is a cancer that mostly develops from the mammary gland's epithelial cells, but it can also be mesenchymal, myoepithelial, or mixed (Salas et al., 2015). Although CMGT can be present as benign or malignant, approximately 40% to 50% of these tumors are malignant, (Sorenmo et al., 2003)

For the case of mammary tumor, surgical excision has remained the mainstay choice of treatment (Lavalle et al., 2012). Mammary gland tumors can be removed surgically using either a lumpectomy or a mastectomy. Surgical excision alone does not effectively treat canine mammary tumour with lymphatic or vascular invasion due to the high rates of recurrence and metastasis (Gilbertson et al., 1983). Using chemotherapy to treat CMGT cases has improved overall survival rates, although there is still no standard chemotherapy regimen for dogs with CMGT (Levi et al., 2021). The first choice of chemotherapy

medicines for canines with CMGT is Doxorubicin. which belongs to the anthracycline class and is made from a secondary metabolite produced by a mutant strain of *Streptomyces peucetius var*. caesius. However, Doxorubicin has a variety of adverse effects, including cardiotoxicity, leukopenia, nausea, vomiting, alopecia, and mucositis. To address this concern, polyethylene glycol doxorubicin with a liposomal formulation (pegylated) was developed (Perez et al., 2002). Commercially known as Doxil®,. The practical utility of pegylated liposomal doxorubicin for treating canine mammary cancer is still poorly understood in veterinary medicine. Given that cardiotoxicity is the most severe adverse effect place a limit on the use of doxorubicin in dogs, the use of Doxil® in CMGT deserves additional research (Zabielska-Koczywas and Lechowski, 2017).

Other than that, usage of non-steroidal anti-inflammatory medicines (NSAIDs), which prevent the activation of prostaglandins. The cyclooxygenase enzyme comes in two varieties, COX-1 and COX-2, which have analgesic and antipyretic properties, respectively, While COX-2 is induced by proinflammatory cascades and overexpressed in a variety of cancers, including mammary carcinoma, COX-1 is constitutively expressed in most tissues and controls multiple physiological activities, such as increasing gastrointestinal integrity and platelet aggregation (Ustun Alkan et al., 2012). According to Queiroga et al. (2011), the COX-2 enzyme was shown to be expressed in CMGT, and a high level of COX-2 enzymes was linked to a bad prognosis because it stimulated tumor angiogenesis, increasing vascular density and tumor growth. COX-2 inhibitors are gaining a lot of interest as a treatment that can halt the growth of tumors. It has been shown that dogs with CMGT overexpress COX-2. Thus, according to Souza et al. (2009) treatment of CMGT dogs using COX-2 inhibitors such as piroxicam, showed a desirable clinical response to the treatment. Other C0X-2 inhibitors include Mavacoxib and Firoxocib also showed positive response toward CMGT treatment.

Recently, Chitosan, a naturally occurring polysaccharide that is present in a variety of shellfish, including prawns, crabs, and crayfish has been reviewed and intensively utilized in biomedical application, in cancer therapy, chitosan has multifaceted applications, such assisting gene in gene delivery and chemotherapeutic delivery and as an immunoadjuvant for vaccines. Due to targeted distribution into the cancer and sustained release, certain therapeutic agents conjugated with chitin or chitosan derivatives have shown excellent anticancer potency with fewer side effects than the original drugs (López-Barrera et al., 2021). Chitosan may also build up in the tumor site, trigger M1 macrophage polarisation, and change the immunosuppressive tumor microenvironment to an immune supportive state, all of which have anti-tumor effect and enhance the effectiveness of cancer immunotherapy (Zeng et al., 2021). Besides, chitosan also can trigger innate immune response (Liang et al., 2021). Last but not least, chitosan itself has the potential to suppress tumor-induced angiogenesis, tumor cell proliferation, and tumor spread. In previous *in vitro* study, chitosan nanoparticles were able to show cytotoxic effects on cancer cells while having minimal toxicity on normal cells (Zoe et al., 2023)

Asteraceae family member Gynura Procumbens (Lour.) Merr. is a common medicinal plant found in tropical Asian nations including China, Thailand, Indonesia, Vietnam, and Malaysia. Because G. procumbens has been used in traditional medicine to cure a variety of illnesses and diseases both systemically and topically, it is known in Malay as Sambung Nyawa, which translates to "prolongation of life" (Krishnan et al., 2015). An extensive range of biological activity, including antibacterial, anti-inflammatory, anticancer, antioxidant, organ protecting, antihypertensive, cardioprotective, and many more, have been demonstrated by prior research on G. procumbens (Tan et al., 2016*). G. procumbens* has also shown promise in the prevention

of breast cancer. It has been demonstrated to effectively limit the growth of breast cancer and mammary gland epithelial cells. Additionally, another research has demonstrated that treating G. procumbens was effective in lowering the occurrence of tumours in the studied animals (Meiyanto et al., 2007; Hew et al., 2013; Gofur et al., 2015).

With the increasingly population of dogs in the world, together with the prevalence of CMGT, this require Veterinary medicine field to evolve, improve, and consider available options for CMGT treatment so that we can reach toward better health prognosis and welfare of the animal , thus this study aims to explore and demonstrate the potential ability of chitosan and usage of *gynura procumbens* as new modular treatment for canine mammary carcinoma by studying its physicochemical characterisation and cytotoxic effect toward canine mammary tumor cells.

2.0 PROBLEM STATEMENT

Canine mammary gland tumor is a most common tumor among female dogs and becomes a serious illness to the dogs. Like other types of cancer in humans, CMGT also has the same issues of side effects of treatment and relapse. The surgical removal of a tumor principally able to control cancer from metastasising but not to cure and total removal of cancer cells from the body, Chemotherapy such doxorubicin in other hand associates with severe side effects such as including cardiotoxicity, leukopenia, nausea, vomiting, alopecia, and mucositis during the course of treatment. Therefore, there is a need to find a new therapy not only to treat the current condition and underlying pathophysiology but to also prevent the recurrence of the disease. Considering the idea of nanomedicine technology, hence, discovery of antitumor using chitosan nanoparticles for the CGMT treatments need to be elucidated and, *Gynura procumbens*, a plant which has abundant of biological activity include anticancer properties was used in this study to

recapitulate its anticancer properties and uses a example model for active ingredient in nanocarrier application of chitosan of this experiment. The findings from this proposed study would providing a new paradigm towards the effectiveness of chitosan nanoparticle as antitumor in Veterinary Medicine.

3.0 RESEARCH QUESTION

i. What is the minimum inhibitory concentration (IC_{50}) of chitosan nanoparticles that would be able to kill tumor cells, *in vitro*?

ii. Would the chitosan nanoparticle be able to inhibit the growth of cancerous cells?

4.0 RESEARCH HYPOTHESIS

i. Chitosan nanoparticle would exhibit a potent antitumor effect against canine mammary gland tumor (CMGT) using mouse 4T1 breast cancer line model

5.0 RESEARCH OBJECTIVES

i. To elucidate the physicochemical characteristic of chitosan nanoparticles formulation

ii. To determine *in vitro* cytotoxicity effects of chitosan nanoparticles formulation against mammary tumor cell line

iii. To determine the phytochemical constituent of *Gynura procumbens* extract.

6.0 LITERATURE REVIEW

6.1 Canine Mammary Gland Tumor

Globally, the most prevalent malignancy in intact bitches (Canis familiaris) is canine mammary gland tumor (CMGT) (Goldschmidt et al., 2011). A later, more deadly, and more malignant variant has been associated with CMGT (Sorenmo et al., 2000; Kristiansen et al., 2013). Males can also get CMGT, however this is very uncommon, it is 66 times less likely to occur and there aren't many instances of it happening in this population (Saba et al., 2007). As to the findings of Vascellari et al. (2016), out of 100,000 canines, 250 were diagnosed with CMGT, accounting for 56% of all canine neoplasm cases. There is a higher occurrence in some breeds than others, including German Shepherds, Poodles, English Spaniels, and English Setter Spaniels

6.2 Mouse 4T1 Breast Cancer Cell Line

4T1 breast cancer cell lines are a murine transplantable tumor that is highly tumorigenic, invasive and can spontaneously metastasize from the primary tumor in the mammary gland to multiple distant sites including lymph nodes, blood, liver, lung, brain, and bone. The 4T1 breast cancer cell line is a suitable *in vitro* animal model for human breast cancer due to the similarities in characteristic between them (Pulaski *et al*., 2001). Similarly, dogs have been proposed as spontaneous animal models of human breast cancer, based on clinicopathologic similarities between canine and human mammary cancer (Nguyen *et al*., 2018). Thus, positive results obtained from cancer studies conducted on 4T1 breast cancer cell lines can be extrapolated directly to human breast cancer and indirectly to canine invasive mammary carcinoma with further investigations.

6.3 Limitation of Current Cancer Therapy

Few studies reported the use of chemotherapy as adjuvant therapy did not affect a better prognosis for CMGT (McNeill et al., 2009). Cancer patients that undergo current cancer therapy such as chemotherapy and radiotherapy primarily experience side effects. The side effects include hair loss, nausea, anemia, skin irritation, nephrotoxicity and infertility (Jung et al., 2014). Chemotherapy is a non-specific cancer therapy with high toxicity levels and it also develops drug-resistant at times. Chemotherapy is toxic as there is limited aqueous solubility due to the diluent used to make up the treatment. It also lacks specificity as current chemotherapy drugs kill rapidly dividing cells which include both cancer cells and also normal healthy cells such as bone marrow and immune cells (Lim et al., 2011).

6.4 The Directed Effects of Chitosan and its Derivatives on Cancer Progression

Chitosan is a suitable polysaccharide with a low level of toxicity. It has been claimed that taking chitosan or oligochitosan can protect the body from oxidative stress brought on by cancer. Their increased penetration qualities are mostly responsible for their anti-metastatic effect (Amirani et al., 2020). A higher chitosan content inhibited the migration of MDA-MB-231 human breast cancer cells (Nam and Shon, 2009). A further method by which chitosan functions as an anticancer agent is through improving the biodistribution of medicines. Chitosan's enhancement of cell permeability and drug retention duration to low toxicity is what causes the drug to accumulate in tumor cells (Adhikari and Yadav, 2018). In Ehrlich ascites tumor (EAT) harbouring human breast cancer cell lines, self-assembled microparticles from chitosan (SAMC) display tumor growth suppression in model mice. Additionally, SAMC reduces ascites' VEGF output, which also results in less neovascular development. As a result, SAMC may be yet another possible dietary supplement that has anticancer properties (Punarvasu and

Prashanth, 2022). Chitosan may be modified by adding amino, acetamido, and hydroxy groups to produce a variety of derivatives that have exceptional anticancer potential and improved solubility. It has been noted that chitosan and its many derivatives have anticancer action through numerous cellular apoptotic pathways (Jiang et al., 2015).

6.5 Chitosan Based Delivery System

Chemotherapeutic medicines have been delivered to tumor locations using a variety of nanocarriers. According to Wang et al. (2021) these nanocarriers can typically circumvent the immune surveillance system, achieve target selectivity, enter the inside of malignant cells, avoid endosomal entrapment, and release the medications over time.

Due to its naturally advantageous qualities, chitosan has gained popularity as a potential drug carrier and has been extensively used in recent years (Dousti et al., 2021). To maintain long-term therapeutic levels of medication concentration in the targeted location, administration duration, and reduce side effects for disease therapy, tremendous efforts have been undertaken. The development of more efficient treatment strategies that may be used more safely and with fewer side effects is directly tied to the hunt for innovative controlled drug release mechanisms. One of the most useful natural biopolymers with high biocompatibility and biodegradability is chitosan, which is frequently employed in the pharmaceutical industry.

6.6 Chemotherapeutic Drugs Delivery

Over the past almost 50 years, traditional chemotherapy agents like doxorubicin, paclitaxel, cisplatin, and others have significantly advanced the field of cancer treatment. However, the very serious adverse effects that these medications also produce limit their extensive clinical usage in the treatment of cancer. Numerous studies have been carried out to address these serious side effects of chemotherapy medications. To reduce the

negative effects, DDSs are used with controlled, targeted, and prolonged drug release. A relevant DDS is a chitosan-based nanoparticle that may enclose and distribute several anticancer medications to certain tumor tissues (Ryu et al., 2020). Chemotherapeutic medicines can be covalently bonded to hydrophilic polymers in addition to being encapsulated, and these nanoparticles are known as polymer drug conjugates. Due to the cancer site-specific distribution and sustained release properties of some anticancer drugs, the conjugates show excellent anticancer effects with much milder side effects than the original drug. They also selectively accumulate in tumors and prolong retention time in the blood circulation.

6.7 Gynura Procumbens

Gynura procumbens is a widely known plant utilized in traditional medicine which belongs to the Asteraceae family and many locals from Asian nations frequently employ the benefits of this plant to treat diabetes, cancer, hypertension, inflammation, fever, and skin diseases (Amin et al., 2021). *Gynura procumbens* was formerly known as "Sambungnyawa," a plant whose name also implies everlasting life. This plant can be eaten on a regular basis as raw or cooked. They taste mildly raw, have little Flavors, and faintly pungent fragrance (Adeib Idris et al., 2019). Recent findings have suggested that *Gynura procumbens* holds the potential to be an alternative treatment for persistent nonhealing wounds, particularly diabetic wounds (Sutthammikorn et al., 2021) by analysing its extract's ability to speed up the wound recovery process in rats on both macroscopic and microscopic levels (Zahra et al., 2011). Previous findings reported that GP extracts can accelerate the healing of chronic wounds via angiogenesis which promotes cell migration and proliferation with comparable safety profile in vivo in mice even after long-term used *(*Sutthammikorn et al. 2021).

6.8 Polyphenols

Polyphenols are the major group of phytochemicals extensively found in plant-based foods. Throughout the decades, numerous potential health effects of polyphenols were documented for different diseases such as cancer, inflammation, neurodegenerative and cardiovascular diseases, type 2 diabetes, and obesity. Plant polyphenols are secondary metabolites possessing one or more hydroxyl groups that attach to one or more aromatic rings. Myriad of polyphenolic compounds, including the ones that are edible, have been discovered in higher plants. Flavonoids and non-flavonoids are the two main categories of plant polyphenols. *Gynura procumbens* contains flavonoids such as apigenin, asrutin, myricetin, quercetin, and kaempferol as well. These flavonoids are thought to be excellent antioxidant agents after phenolic compounds. Both astragalin and kaempferol-3-O-rutinoside are deemed as potent antioxidants (Timotius et al., 2021).

Fascinatingly, antioxidants are helpful in the healing process for wounds. Antioxidants prevent tissues from oxidative damage, which notably accelerates wound healing. Through antioxidant activity of *Gynura procumbens*, lipid peroxidation due to oxidative stress can be reduced and collagen fibre strength can be intensified. Ergo, topical dosage forms containing *Gynura procumbens* extract have the prospect to hasten wound recovery (Timotius et al., 2021)

Polyphenols extracted from *Gynura procumbens* are believed to portray an antibacterial crucial function against pathogenic microbes (Timotius et al., 2021). For instance, the availability of tannins and saponins permits potent antibacterial properties (Amin et al., 2021). Herbal remedies such *as Gynura procumbens* are widely employed as a traditional form of treatment or illness prevention since infectious diseases exert such a heavy cost on public health. On top of that, a different study stated that these phenolic compounds can assist in reducing inflammation via the modulation of signalling pathways associated with inflammation and oncogenesis (Kim et al., 2021). Chlorogenic acid is one

of them and is the main active ingredient in *Gynura procumbens* featuring high anti-inflammatory impacts (Cao et al., 2022).

6.9 Nanoparticles based chitosan

In recent decades, chitosan-based nanoparticles—also known as chitosan nanoparticles, ChNPs, have drawn a lot of attention as promising materials. ChNPs are excellent candidates to be nanocarriers. They have the capacity to encapsulate medications and active ingredients and deliver them to a particular location within the body, resulting in a controlled release. Chitosan is derived from the shells of crustaceans, like crabs and prawns.

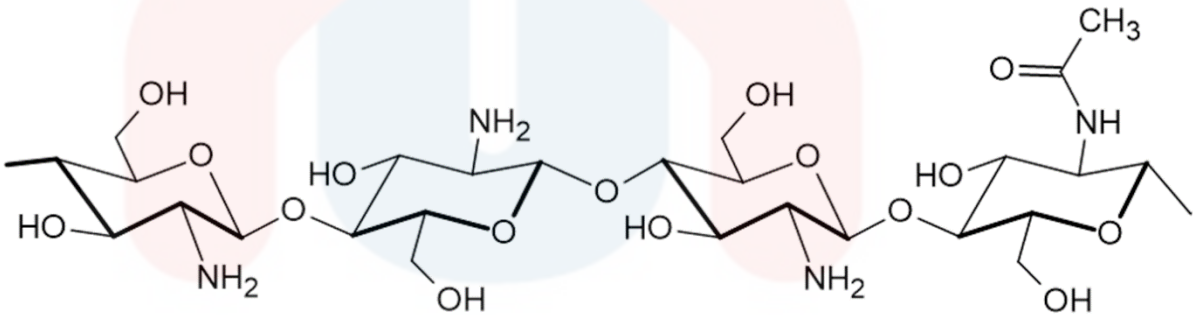


Figure 1: Scheme presenting chemical structure of chitosan

7.0 STUDY METHOD

7.1 Plant extraction

1kg of dried and grinded *G.Procumbens* leaves were collected from a supplier in Perak. in the laboratory, the grinded leaves are then extracted using Soxhlet apparatus and further evaporated to remove the solvent using the rotary evaporator. The extract is then left in the freeze dryer for 24 hours.

Figure 2:Shows the leaves of G.procumbens

Figure 3: grinded G.Procumbens leaves from supplier

Figure 4:Extraction using Soxhlet apparatus

Figure 5: Shows Rotary evaporator used for removal of solvents from samples

7.2 Phytochemical Screening

7.2.1 Qualitative Test

All qualitative tests are described according to Samraj & Rajamurgugan (2017) with light modifications.

7.2.1.1 Test for Alkaloid

Wagner's reagent was added in addition to a few drops of *G. procumbens* extract, and the appearance of a reddish-brown precipitate shows the alkaloids' composition. Samraj & Rajamurgugan (2017)

7.2.1.2 Test for Tannins

Approximately 1 mL of *G. procumbens* leaves extract will be added with a little amount of 10% alcoholic ferric chloride in order to perform a test for tannins. Tannins can be detected by the development of solutions with a brownish-blue or bluish-black tint. Samraj & Rajamurgugan (2017)

7.2.2 Quantitative Test

Both Total Phenolic Contents and Total Flavonoid Contents are to be determined according to Wulandari et al., (2017) with some modifications.

7.2.2.1 Determination of Total Phenolic Contents

During the present research, the Folin-Ciocalteu reagent (FCR) will be utilized to quantify the total phenolic content of the *G. procumbens* leaves extract. The FCR was six times serially diluted before being left to stand at ambient temperature for five minutes. After adding 2 or 3 drops of the extract to 1.5 mL of reagent, 1.5 mL of Na2CO3 solution was added. A 725 m spectrophotometer was used to measure the absorbance after the

mixture was incubated for 90 minutes. The result is expressed as mg GAE/g or milligrams of gallic acid equivalents per gram of material. Wulandari et al., (2017)

7.2.2.2 Determination of Total Flavonoid Contents

The colourimetric method was used with quercetin as a standard in determining the Total Flavonoid Contents (TFC). Different concentrations of standard quercetin and ethanol were prepared in test tubes. 1 ml of *G. procumbens* leaves extract was put in a test tube with an addition of 0.33 ml of 5% $NaNO_2$ solution. Next, 0.5 ml of 10% $AlCl_3$ solution was added after 6 minutes. The mixture was left for 5 minutes and 0.5 ml of 1M NaOH was then added. Then, the mixture is mixed with a vortex mixer and the absorbance of the mixture was observed immediately by using a 510nm spectrophotometer. The result was given in mg quercetin equivalents (mg QE/g) per 1 g of dried *G. procumbens* leaves. Wulandari et al., (2017)

7.3 Preparation & Physicochemical Characterization of Chitosan Nanoparticle-*Gynura Procumbens*

The synthesis of amphiphilic chitosan was performed according to method described by (Xiao et al., 2011) with a slight modification.1 g of chitosan was dissolved into 98 ml of 1% acetic acid to achieve 0.1% solution (w/v) on magnetic stirrer for 3 hours until transparent. The pH of the solution was adjusted and stabilized between 3 and 4 using the 4N NaOH solution. Then, 0.1 g Sodium Tripolyphosphate (TPP) was dissolved into 98 ml of deionized water using magnetic stirrer at ambient temperature to achieve 0.1% TPP solution. From 100 ml of chitosan solution, 5 ml was pipetted into a beaker. 0.2 ml of TPP was added into 5 ml of chitosan solution dropwise while being stirred using a magnetic stirrer for 60 minutes. 50 mg extract powder was dissolved in 5 ml of distilled water to achieve 1% concentration. Then 1 ml of 1% *Gynura procumbens* extract was inserted into Chitosan-TPP solution. The solution was mixed for 30 minutes with magnetic stirrer. Then, chitosan nanoparticle created was characterized by using dynamic light scattering (DLS) to measure the particle size, polydispersity index (PDI) and zeta

potential. Sample was diluted with particle -free distilled water, 100 folds and will be sonicated for twenty (20) minutes and prior placed on the grid and allowed to dry at room temperature.

7.4 *In Vitro* cytotoxicity Assay

7.4.1 Culture Medium, Cell line preparation and MTT Assay

Mouse mammary cancer cell lines(4T1) were used for this study. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) culture media. As a complete growth medium (CGM), 10% fetal bovine serum (FBS) and 1% antibiotic antimycotic (Gibco, Thermo Fisher Scientific) will be added to all the media. The cells were cultured with 5% carbon dioxide (CO2) in an incubator at 37 C after gradually defrosting from liquid nitrogen to an 80 C freezer and finally a 37 C water bath. Daily observation of the cells' viability, proliferation, and 70% confluency was made.

With 0.25% trypsin the confluent 4T1 cells were separated and collected. In 96-well plates, 100 µL of medium containing $1X10^4$ cells was seeded. To enable cell adhesion to the bottom of the well plates, the plate was incubated for a whole night. Old medium was taken out after an overnight incubation. The experiment was carried out in triplicate on one plate with different concentration of treatment substance µg/ml of the cells with *Gynura procumbens* extract,Chitosan and Chitosan-*Gynura procumbens* extract, and untreated cell. The experiment was repeated thrice to ensure its validity. The plates were incubated for another 72 hours. The old media was removed and replaced with 100µL of new medium after the treatment duration of 72 hours. Each well receives 20 µL of filtered MTT solution (5 mg/mL PBS). The plates once again were incubated for 3 hours at 37 degrees C with 5% CO2 after being covered in aluminium foil. After

carefully aspirating 110 µL of media from each well, 100 µL of DMSO was added and well mixed using a pipette The plates were further incubated for another 10 minutes. After 10 minutes of incubation, the optical density (OD) was measured at 570 nm with a spectrophotometer (Infinite M200 PRO).

The percentage of cell viability (%) was calculated using the formula

$$Cell\ viability\ (\%) = \frac{mean\ OD\ of\ treated\ cell - mean\ OD\ of\ blank}{mean\ OD\ of\ untreated\ cell - mean\ OD\ of\ blank}\ x\ 100\%$$

Cytotoxic effects on 4T1 cells were measured as IC_{50} in comparison to untreated cells

7.5 Cell Scratch Migration Assay

The procedure was conducted based on scratch wound healing assay study by (Martinotti & Ranzato, 2019) with slight modification. In the migration assay 5 x 10^4 cells were seeded in each of the 6 well culture plates. The cells were detached from tissue culture dish using Trypsin/EDTA, the 6-well culture plate with 2ml warmed media added to each well, then the cells were seeded into 6-well tissue culture, and 70% confluency was achieved after 24h growth in incubator. Then the cell layer in each well was scraped in a straight line using a 100µL pipette tip. After scratch was done the monolayer cell layer of each well was then gently washed with 1ml PBS then replenished with 1 ml of fresh medium, another 1 ml was added with GP extract, Chitosan, & Chitosan-extract treatment respectively. One well was left untreated as negative control; images of each plate were taken under stereo microspore before incubation. Then, the 6-well growth plate was then left in the incubator in 72 hours and the cell was observed under microscope and the images of each plate were taken again.

7.6 Statistical Analysis

All the percentages of cell viability were expressed as mean (n = 3) per plate (standard deviation) and differences among treated and untreated cells were analysed using one-way ANOVA followed by Dunnett's multiple comparison test. The test was considered statistically significant when $P < 0.05$ as compared to untreated cells and GraphPad Prism software 5.0 was used to analyse all statistical tests.

8.0 RESULT

8.1 Phytochemical Screening: Qualitative Analysis

Two phytochemical tests were conducted and the results were shown in table 1. GP extracts were positive for Alkaloids and Tannins test. The result in Table 2 below revealed the presence of alkaloids with the formation of reddish-brown precipitate, as for tannins, there was a brownish or blue-black coloration

Table 1: Shows the result for qualitative analysis in the GP sample.

No	**Test**	***G.Procumbens* extract**		
		Trial 2	**Trial 2**	**Trial 3**
1	**Alkaloids**	+	+	+
2	**Tannins**	+	+	+

(+) indicates positive result, (-) indicates negative result

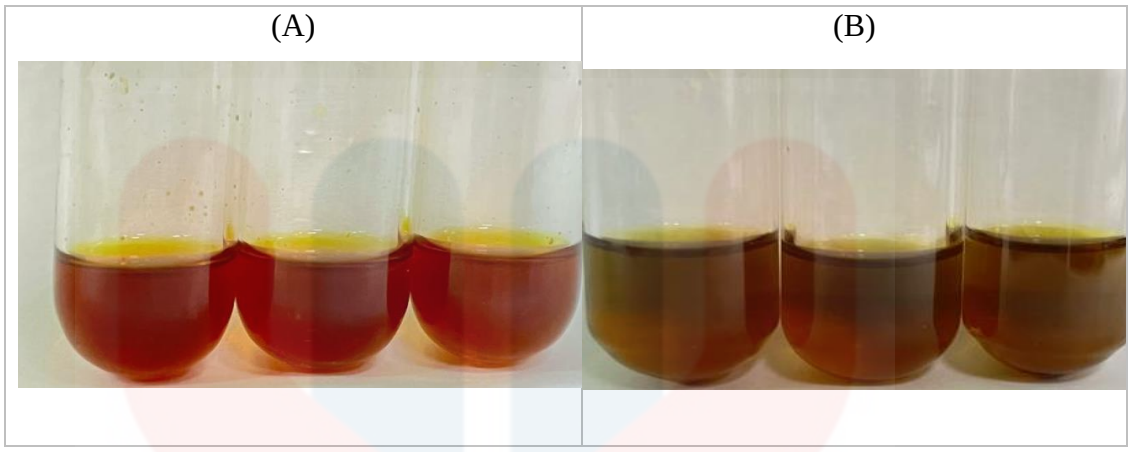


*Table 2 **:** Presence of Alkaloids(A) ; Tannins (B)*

.

8.2 Phytochemical Screening: Quantitative Analysis

8.2.1 Total Phenolic Content

. An equation obtained from the calibration curve of standard gallic acid (Figure) is y= 0.0197x + 0.3743, where R^2= 0.9503. Refer figure 6 and Table 3.

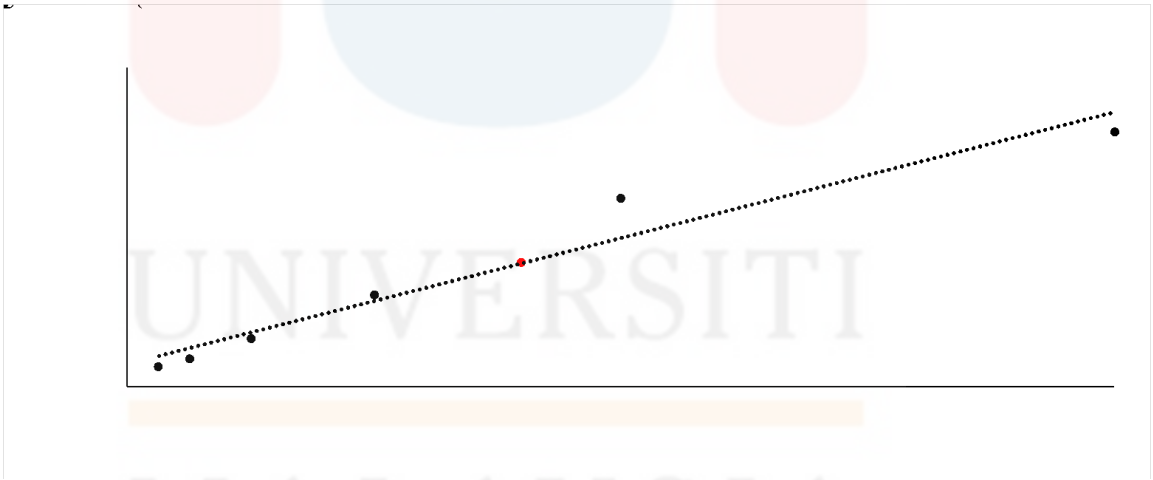


Figure 6:Calibration curve of standard gallic acid and GP sample

Table 3: Total Phenolic Content (TPC) Determination

Absorbance	Total Phenolic Content (mg of GAE/g)
1.960	79.808

The TPC *of G.Procumbens* leaves obtained in this experiment was 79.808 mg of GAE/g

8.2.2 Total Flavonoid Content

An equation obtained from the calibration curve of standard quercetin is y =0.0223x + 2.2483, where R^2 = 0.8122. Refer to figure 7 and Table 4.

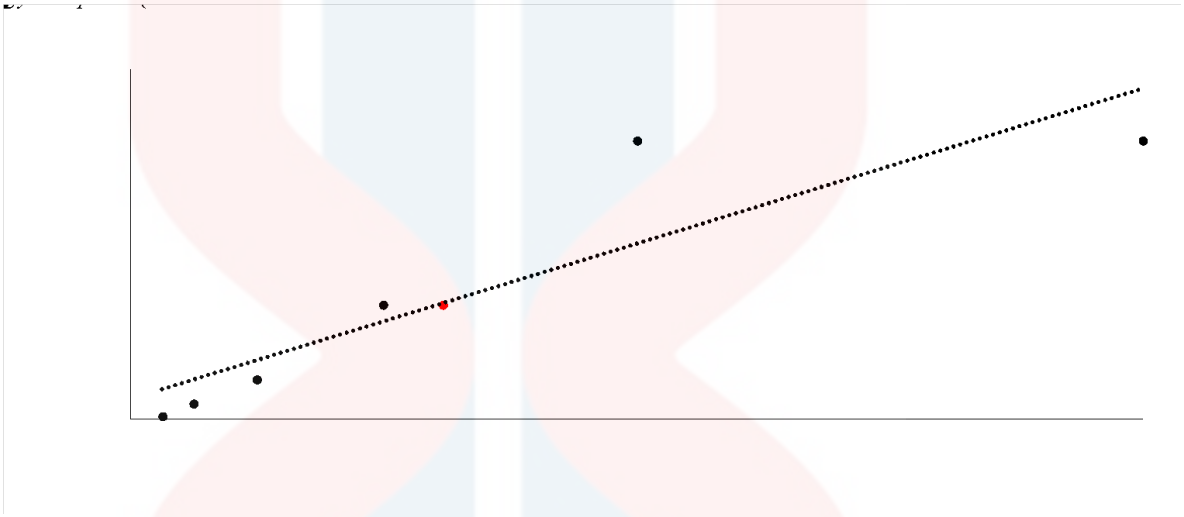


Figure 7: Calibration curve of Quercetin standard and GP sample

Table 4: Total Flavonoid Content (TFC) Determination

Absorbance	Total Flavonoid Content (mg of QE/g)
1.634	61.621

The TFC of G. Procumbens leaves extract obtained in this experiment was 61.621 mg of QE/g

8.3 Physicochemical Characteristic of Chitosan Nanoparticle-TPP

The physiochemical characteristic of chitosan nanoparticle in term of Size, PDI and Zeta potential was tabulated. Refer to table 5

Chitosan Concentration (µg/ml)	TPP Concentration (µg/ml)	Size (nm)	PDI	Zeta potential (mV)
10	10	340±7	0.3	14±1

Table 5: shows the result for physicochemical characteristics of chitosan nanoparticles using ionic gelation method with TPP, the size was 340nm, with PDI of 0.3 and Zeta potential 14.

8.4 MTT Assay

8.4.1 Effect of, GP Extract, Chitosan, Chitosan Based Nanoparticles *with G. Procumbens* extract On 4T1 Cells

The cell viability of 4T1 against the treatments were calculated and a line chart was made. The cell survivability (%) of 4T1 cell line following treatment with *G. procumbens* extract, Chitosan solution and Chitosan with *G.Procumbens* extract for 72 h, respectively was shown in Figure 8 Refer to figure 8

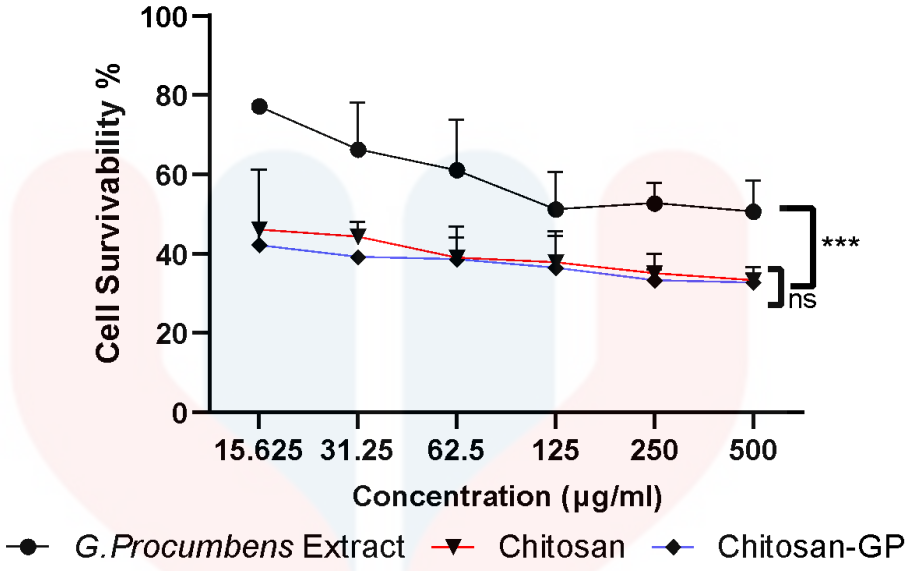


Figure 8:Percentage of mean cell viability % in 96-well plate of 4T1 cell line after 72 hours of treatment with GP extract, Chitosan & Chitosan-GP. Every point denotes the mean (n =3) of a triplicate sample. P value 0.0010,0.0015 between GP extract and Chitosan, Chitosan-extract while 0.0868 between Chitosan & Chitosan-GP extract

.

The findings showed that for GP extract treatment, 15.625 µg/ml Concentration of GP extract showed the highest percentage of cell viability of 77.19 % while 500 µg/ml concentration showed the lowest percentage of cell viability of 50.66%. For Chitosan treatment,15.625 µg/ml Concentration shows the highest percentage of cell viability of 46.12% while 500µg/ml concentration showed the lowest percentage of cell viability of 33.32%. For Chitosan-GP treatment,15.625 µg/ml Concentration shows highest percentage of cell viability of 42.18% while 500µg/ml concentration showed the lowest percentage of cell viability of 32.82%

Figure (9,10,11) below represents the percentage of cell inhibition % of GP extract, Chitosan, Chitosan-GP treatment respectively on a 4T1 cell line after 72 hours of treatment. Highest cell inhibition of 4T1 cells recorded was 49.34% in 500µg/ml treatment of GP extract and the lowest cell inhibition was 22.81% in 15.625µg/ml with

the same treatment. Highest cell inhibition in 4T1 cell recorded was 66.68% against Chitosan treatment 500 µg/ml and the lowest cell inhibition was 53.88% in 15.625 µg/ml treatment concentration Lastly for Chitosan-GP treatment, highest cell inhibition recorded was 67.180% in 500 µg/ml while the lowest was 57.82% in 15.625 µg/ml of the treatment.

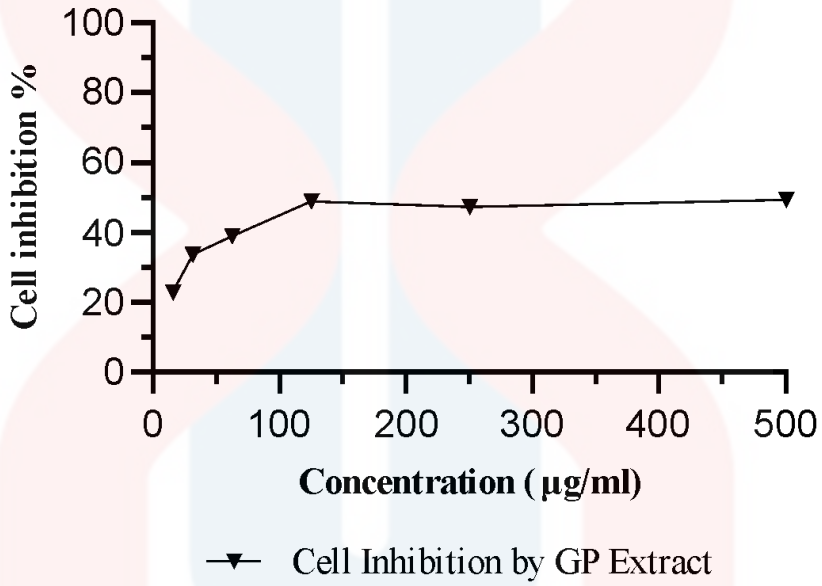


Figure 9: shows Inhibition % of 4T1 cells by the GP extract treatment after 72 hours

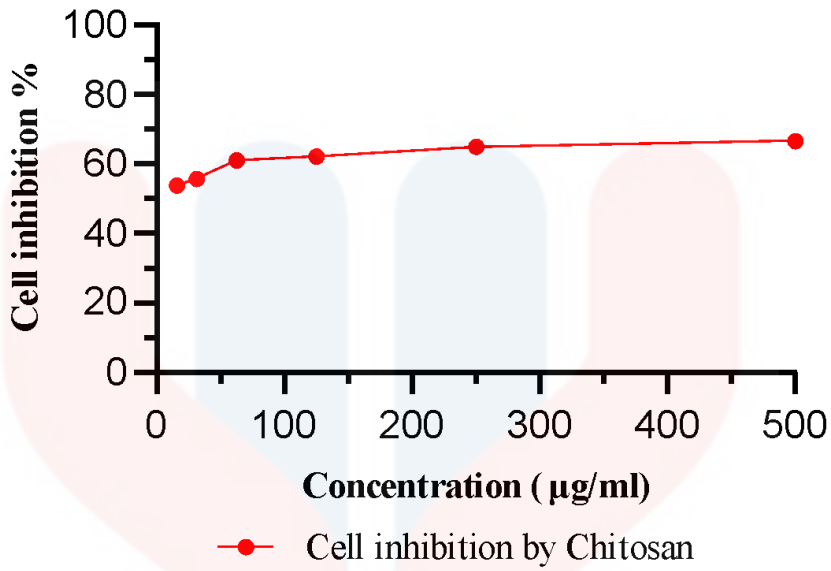


Figure 10 : Shows Inhibition % of 4T1 cells by Chitosan treatment after 72 hours.

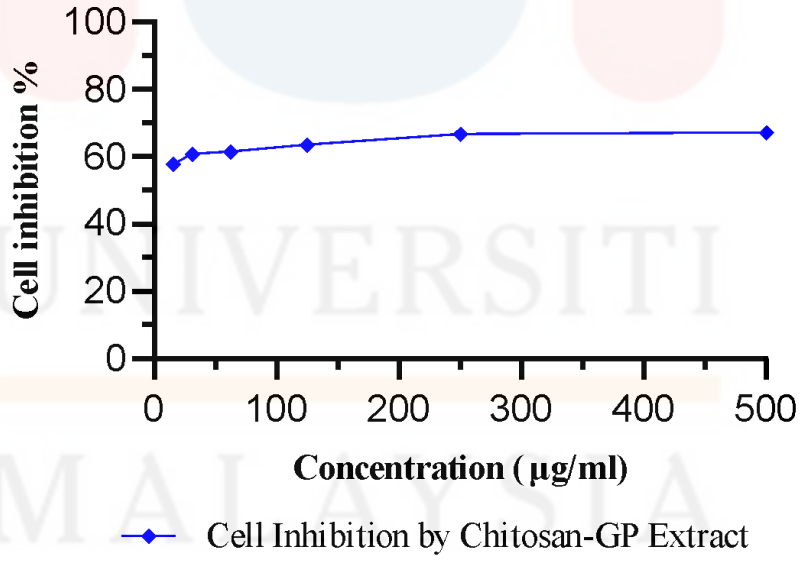


Figure 11 : Shows cell inhibition % of 4T1 cells by Chitosan-GP extract treatment after 72 hours.

Then the mean percentage (%) of cell viability of 4T1 (n=3) Cells after 72 hours of treatment using GP extract, Chitosan, Chitosan-GP extract was tabulated into tables.

Table 6: The mean percentage (%) of cell viability of 4T1 cell lines after 72 Hours of treatment with GP extract.

Concentration (µg/ml)	15.625	31.25	62.5	125	250	500
Cell Viability %	77.20	66.25	61.10	51.18	52.73	50.66

Based on the findings in table 4 ,500µg/ml has the lowest mean of cell viability which is 50.66% thus, the inhibitory concentration (IC_{50}) of the GP Extract treatment was border on or not slightly more than 500µg/ml

Table 7: The mean percentage (%) of cell viability of 4T1 cell lines after 72 Hours of treatment with Chitosan

Concentration (µg/ml)	15.625	31.25	62.5	125	250	500
Cell Viability %	46.12	44.27	38.96	37.76	35.03	33.32

Based on findings on table 5, the lowest concentration 15.625 µg/ml tested in this experiment had 46.12% of cell viability suggesting that the inhibitory concentration (IC_{50}) of Chitosan treatment was lower than 15.625µg/ml

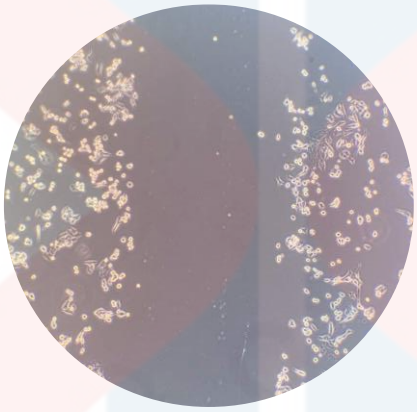
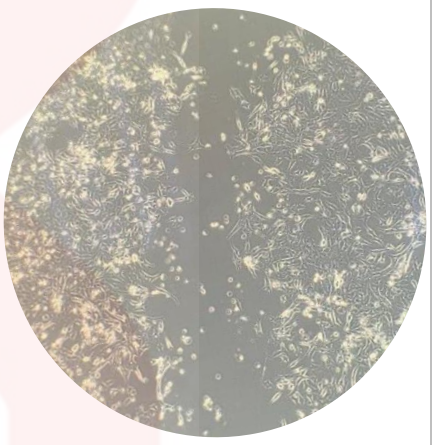
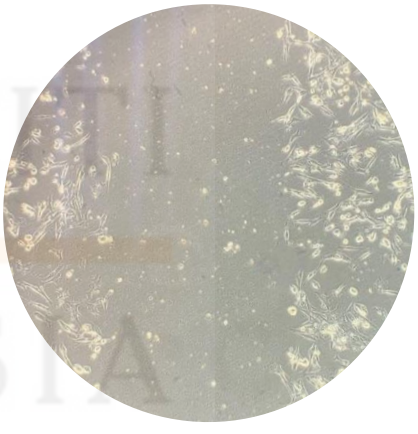
Table 8: The mean percentage (%) of cell viability of 4T1 cell lines after 72 Hours of treatment with Chitosan-GP Extract

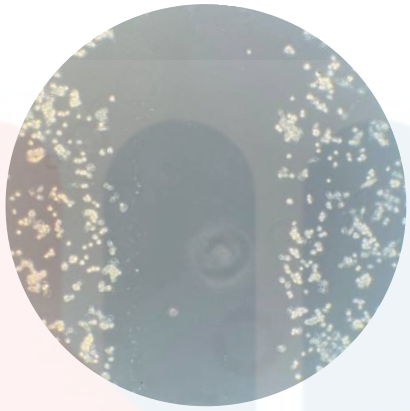
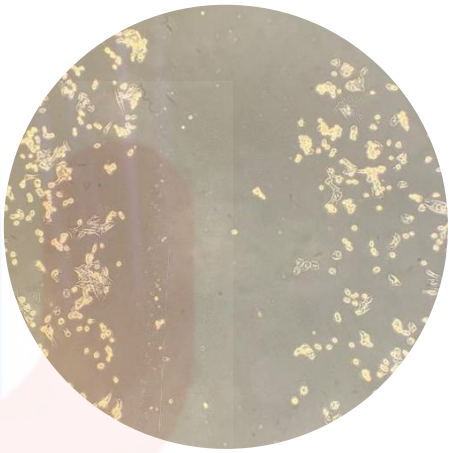
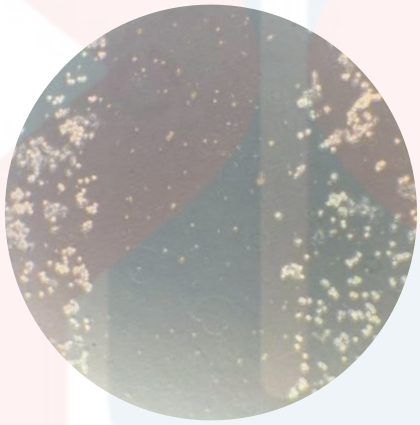
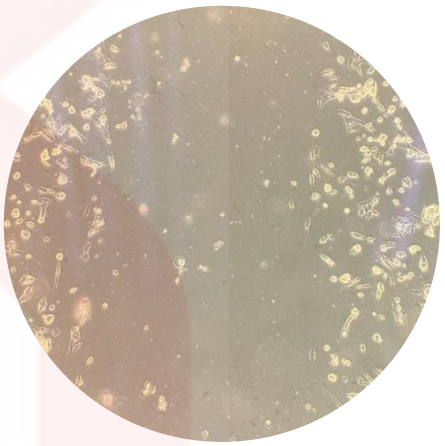
Concentration (µg/ml)	15.625	31.25	62.5	125	250	500
Cell Viability %	38.56	36.42	33.33	32.82	33.22	32.82

Based on findings on table 6, the lowest concentration 15.625 µg/ml tested in this experiment had 36.56% of cell viability suggesting that the inhibitory concentration (IC_{50}) of Chitosan treatment was lower than 15.625(µg/ml)

8.5 Cell Scratch Migration Assay

Table 9 : show the effect of treatment against the migration of 4T1 cell after 72 hours

Treatment	0 Hour	72 Hours
Negative		
Extract		

Chitosan		
Chitosan-Extract		

9.0 Discussion

This study was aimed to elucidate the physicochemical characteristic of Chitosan nanoparticles and investigate the cytotoxicity activity of Chitosan functionalized with *Gynura Procumbens* extract against 4T1 Cell lines. Chemoprevention is a mechanistic approach which uses pharmacological agents to inhibit, arrest or reverse carcinogenesis at its earliest stages (Sporn & Suh, 2002). As a consequence, prevention of tumor promotion is typically regarded as a more effective approach in cancer chemoprevention. Currently, research has shifted towards developing superior chemopreventive agents from natural products, especially plants (Yeong et al.,2015). In that stance, this study uses *Gynura Procumben* extract which has been claim to have phytochemical compound such

as flavonoid, phenolic,alkaloids and tannin which can be uses as antitumor agent (Kaewseejan et al., 2015). The study also able to postulate the function of Chitosan nanoparticle which has been believed to possess inhibition properties toward mammary breast cancer (Nam and Shon, 2009)

Gynura Procumben Phytochemical Screening

Phytochemical compounds are secondary metabolites of plants that present either in a mixture or alone in all plant species and are present in varying amounts among different species of plant (Nordin et al., 2017). The outcome of extracted plant phytochemical relies on the inherent characteristic of the plant material, its origin, grade of processing, plant's moisture content and size of particle (Pandey & Tripathi, 2014). In this study qualitative and quantitative phytochemical screening was performed to detect the presence of Phenolic, Flavonoid, Alkaloids and Tannin in the extract. Thus from this study the result reveals that *Gynura procumbens* extract contains Alkaloids and Tannins which shows the change in colour in all triplicate qualitative experiments. Phenolic is a substance that demonstrates potent antioxidant and anticancer properties and it is frequently found in plant-origin food (Tsao, 2010). Flavonoids are a derivative of phenolic that have antitumor, antioxidant, and anti-inflammatory activity. Other than that, tannins also possess antiviral, antimicrobial, antioxidant, antitumor, enzyme-inhibiting, and radical scavenging properties (Kolodziej & Kiderlen, 2005). A phenolic compound is composed of hydroxyl groups that act as electron donors, which directly induce antioxidant activity and inhibit free radicals. In this experiment, gallic acid was used as a standard natural phenolic compound with strong antioxidant The total phenolic content *of G.Procumbens* leaves obtained was 79.808 mg of GAE/g was found to be higher than previous study which was 16.08 mg of GAE/g according to Niwat Kaewseejan et al., (2014). Flavonoid compounds are directly related to the antioxidant activity that

neutralizes free radicals by donating their hydrogen atoms. It is generally known that flavonoids are polyphenolic chemicals with anti-inflammatory, anti-tumor, antiviral, and antioxidative action that may have positive impacts on human health (Chakraborty & Shah, 2011). In this test, quercetin was used as a standard which is a natural flavonoid with potent antioxidant levels. The total flavonoid content of *G. Procumbens* leaves extract obtained was 61.621 mg of QE/g and was found to be higher than the previous study which was 10.33 mg of QE/g according to Niwat Kaewseejan et al., (2014). Many reasons that may contribute to the result achieved in this experiment such as vegetative factors, soil content, and maturity of the plant species upon time of harvest (Sari et al., 2006). The value obtained might vary due to the geographical location, and the maturity of the plant. For the record, the plant sample based on the journal was collected in Thailand whereas the current plant that was used for this experiment was collected in Perak, Malaysia.

Chitosan Nanoparticle Physicochemical Characteristics

The Chitosan nanoparticles were characterized in terms of the size, Polydispersiti indec (PDI) and Zeta potential using Zetasizer (Malvern Instruments, UK), based on the dynamic light scattering (DLS) technique reveals the size of chitosan nanoparticles were approximately 340 nm, and PDI value of chitosan nanoparticle was 0.3. The usual diameter of nanoparticle drug delivery vehicles is between 10 and 200 nm, and they have several potential benefits, such as increased drug efficacy and cytotoxicity (Mitra et al., 2001), improved drug delivery efficiency (Park et al., 2009), and easier penetration of different biological barriers like tumor vasculature and mucosal membrane (Krasnici et al., 2003). Samples with a greater variety of particle sizes have higher PDI values, whereas samples with equally sized particles have lower PDI values. The PDI was utilized as an indication for nanoparticle stability and uniformity of production. The Zeta

Potential of chitosan nanoparticles that was obtained in this experiment was 14 mV. Nanoparticles with zeta potentials of more than +30 mV or less than -30 mV are thought to be strongly cationic and strongly anionic, respectively, those with zeta potentials between -10 and +10 mV are thought to be about neutral. Zeta potential can influence a nanoparticle's propensity to penetrate membranes since the majority of biological membranes are negatively charged. Thus, the findings from this study suggest that the chitosan nanoparticle has been produced from this study had slightly differ from the ideal expected and desirable physicochemical characteristic of the Chitosan nanoparticle however, in spite of this it remains its usability, utility and functionality as antitumor and antitumor drug nanocarrier. Factor that control the size, formation and distribution chitosan nanoparticle obtained via ionic gelation with TPP includes pH, the fraction of primary amino groups, solute concentration and inclusion of purification steps (Masarudin et al.,2015) in which was not discreetly optimized in this study but rather was recapitulating the concentration of the TPP and Chitosan that was described in previous study.

Cytotoxic Effect Against 4T1 cells

The cytotoxicity of the Chitosan and *Gynura procumbens* extract against 4T1 cells was assessed using cell based colorimetric assay, (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) MTT assay. Chitosan, Gynura procumbens extracts, and Chitosan's cytotoxic impact were evaluated using the minimal concentration of extract that provided at least 50% of the survival of cancer cells (IC50). According to Baharum et al. (2014) and Atjanasuppat et al. (2009), there are four types of substances extremely active (IC50 ≤ 20 μg/mL), moderately active (IC50 > 20–100 μg/mL), weakly active (IC50 > 100–1000 μg/mL), and inactive (IC50 > 1000 μg/mL). A pure substance or medicine is deemed powerful if its IC50 value is less than 4 μg/mL (Boik, 2001; Lee, 2005).In our experiment IC_{50} *Gynura Procumbens* extract, Chitosan and Chitosan-GP extract were concluded as slightly higher than 500(μg/ml) for GP extract, (weakly active) and both below 15.625(μg/ml) for Chitosan and Chitosan-GP extract(extremely active).

Cell migration assay was done to study the effect of the treatments toward the migration of the scratched 4T1 cell inside media culture flask. From our experiment all treatment namely GP extracts, Chitosan, Chitosan-GP extract was also able to decrease the cell migration as has been portrayed and showed in *Table 9.* According to a prior study, chitosan can limit the motility of mammary breast cancer. Tumor metastasis from its initial location to distant organs requires migration, and any change in cell movement will stop the metastasis cascade (Nam and Shon, 2009).

10.0 Conclusion

Based on the findings from this experiment indicate that Chitosan and Chitosan-GP treatment possess extremely active cytotoxic substance and were able to demonstrate the cytotoxicity & migration inhibition activity against mouse 4T1 breast cancer cell line while *G.Procumbens Extract* alone has showed weakly active cytotoxic activity. All in all, these substances may be a potential candidate and have the ability to be used and implemented as a new modular treatment for canine mammary gland tumors in the future.

11.0 Recommendations and Future Work

There is still much work and in-depth research that needs to be done in order to establish the uses of Chitosan nanoparticles as an alternative for mammary gland antitumor treatment in animals. In vivo experiment should be carried out in order to study the cytotoxicity effect of *G.Procumbens* extract, Chitosan and Chitosan-GP, as a complete body system of mammary gland tumor induced laboratory animal may show synergistic effect as well when be treated with these substances thus the effectiveness of the treatment can be elucidated and may provide important information for future uses.

Furthermore, in this experiment only 4T1 mammary cancer cells model was used, in future, the test needs to be repeated into the normal healthy cell lined model to concludes its selectivity and efficacy as an antitumor.

Besides that, other choices of active ingredient such as other plant extract with higher known antioxidant contents or available anticancer drugs such doxorubicin may be used in the future to study the efficacy of the treatment using Chitosan nanocarrier technology.

12.0 References

1. Montoya, M., Morrison, J. A., Arrignon, F., Spofford, N., Charles, H., Hours, M. A., & Biourge, V. (2023). Life expectancy tables for dogs and cats derived from clinical data. Frontiers in Veterinary Science, 10, 145.
2. 2.Kim, E., Choe, C., Yoo, J. G., Oh, S. I., Jung, Y., Cho, A., ... & Do, Y. J. (2018). Major medical causes by breed and life stage for dogs presented at veterinary clinics in the Republic of Korea: a survey of electronic medical records. PeerJ, 6, e5161.
3. .Sorenmo, K. U., Rasotto, R., Zappulli, V., & Goldschmidt, M. H. (2011). Development, anatomy, histology, lymphatic drainage, clinical features, and cell differentiation markers of canine mammary gland neoplasms. Veterinary pathology, 48(1), 85-97.
4. Saba, C. F., Rogers, K. S., Newman, S. J., Mauldin, G. E., & Vail, D. M. (2007). Mammary gland tumors in male dogs. Journal of Veterinary Internal Medicine, 21(5), 1056-1059.
5. Vascellari, M., Capello, K., Carminato, A., Zanardello, C., Baioni, E., & Mutinelli, F. (2016). Incidence of mammary tumors in the canine population living in the Veneto region (Northeastern Italy): Risk factors and similarities to human breast cancer. Preventive Veterinary Medicine, 126, 183-189.
6. Baba, A. I., & Câtoi, C. (2007). Mammary gland tumors. In Comparative Oncology. The Publishing House of the Romanian Academy.

7. Spoerri, M., Guscetti, F., Hartnack, S., Boos, A., Oei, C., Balogh, O., ... & Kowalewski, M. P. (2015). Endocrine control of canine mammary neoplasms: serum reproductive hormone levels and tissue expression of steroid hormone, prolactin and growth hormone receptors. BMC veterinary research, 11, 1-10.
8. Salas, Y., Márquez, A., Diaz, D., & Romero, L. (2015). Epidemiological study of mammary tumors in female dogs diagnosed during the period 2002-2012: a growing animal health problem. PloS one, 10(5), e0127381.
9. Sorenmo, K. (2003). Canine mammary gland tumors. Veterinary Clinics: Small Animal Practice, 33(3), 573-596.
10. Lavelle, K., Downing, A., Thomas, J., Lawrence, G., Forman, D., & Oliver, S. E. (2012). Are lower rates of surgery amongst older women with breast cancer in the UK explained by co-morbidity?. British journal of cancer, 107(7), 1175-1180.
11. Gilbertson, S. R., Kurzman, I. D., Zachrau, R. E., Hurvitz, A. I., & Black, M. M. (1983). Canine mammary epithelial neoplasms: biologic implications of morphologic characteristics assessed in 232 dogs. Veterinary pathology, 20(2), 127-142.
12. .Levi, M., Salaroli, R., Parenti, F., De Maria, R., Zannoni, A., Bernardini, C., ... & Sarli, G. (2021). Doxorubicin treatment modulates chemoresistance and affects the cell cycle in two canine mammary tumor cell lines. BMC Veterinary Research, 17, 1-15.
13. .Perez, E. A., Geeraerts, L., Suman, V. J., Adjei, A. A., Baron, A. T., Hatfield, A. K., ... & Ingle, J. N. (2002). A randomized phase II study of sequential docetaxel and doxorubicin/cyclophosphamide in patients with metastatic breast cancer. Annals of oncology, 13(8), 1225-1235.
14. .Zabielska-Koczywąs, K., Dolka, I., Król, M., Żbikowski, A., Lewandowski, W., Mieczkowski, J., ... & Lechowski, R. (2017). Doxorubicin conjugated to glutathione stabilized gold nanoparticles (Au-GSH-Dox) as an effective therapeutic agent for

feline injection-site sarcomas—chick embryo chorioallantoic membrane study. Molecules, 22(2), 253.

15. .Üstün Alkan, F., Üstüner, O., Bakırel, T., Cınar, S., Erten, G., & Deniz, G. (2012). The effects of piroxicam and deracoxib on canine mammary tumor cell line. The Scientific World Journal, 2012.

16. De Nardi, A. B., Raposo, T. M. M., Huppes, R. R., Daleck, C. R., & Amorim, R. L. (2011). COX-2 inhibitors for cancer treatment in dogs. Pak Vet J, 31(4), 275-9.

17. .López-Barrera L. D., Díaz-Torres R., Martínez-Rosas J. R., Salazar A. M., Rosales C., Ramírez-Noguera P. (2021). Modification of Proliferation and Apoptosis in Breast Cancer Cells by Exposure of Antioxidant Nanoparticles Due to Modulation of the Cellular Redox State Induced by Doxorubicin Exposure. Pharmaceutics 13 (8), 1251

18. .Zeng Z., Liu Y., Wen Q., Li Y., Yu J., Xu Q., et al. (2021). Experimental Study on Preparation and Anti-tumor Efficiency of Nanoparticles Targeting M2 Macrophages. Drug Deliv. 28 (1), 943–956.

19. .Liang X., Li L., Li X., He T., Gong S., Zhu S., et al. (2021). A Spontaneous Multifunctional Hydrogel Vaccine Amplifies the Innate Immune Response to Launch a Powerful Antitumor Adaptive Immune Response. Theranostics 11 (14)

20. Pulaski, B. A., & Ostrand-Rosenberg, S. (2000). Mouse 4T1 breast tumor model. Current protocols in immunology, 39(1), 20-2.

21. Nguyen, F., Peña, L., Ibisch, C., Loussouarn, D., Gama, A., Rieder, N., ... & Abadie, J. (2018). Canine invasive mammary carcinomas as models of human breast cancer. Part 1: natural history and prognostic factors. Breast cancer research and treatment, 167, 635-648.

22. Tan, E. Y., Yan, M., Campo, L., Han, C., Takano, E., Turley, H., ... & Fox, S. B. (2009). The key hypoxia regulated gene CAIX is upregulated in basal-like breast

tumors and is associated with resistance to chemotherapy. British journal of cancer, 100(2), 405-411.

23. Lee, A., Lim, W., Moon, B. I., Paik, N. S., Koh, S. H., & Song, J. Y. (2011). Chemotherapy response assay test and prognosis for breast cancer patients who have undergone anthracycline-and taxane-based chemotherapy. Journal of breast cancer, 14(4), 283-288.

24. Amirani, E., Hallajzadeh, J., Asemi, Z., Mansournia, M. A., & Yousefi, B. (2020). Effects of chitosan and oligochitosans on the phosphatidylinositol 3-kinase-AKT pathway in cancer therapy. International journal of biological macromolecules, 164, 456-467.

25. Nam, K. S., & Shon, Y. H. (2009). Suppression of metastasis of human breast cancer cells by chitosan oligosaccharides. Journal of microbiology and biotechnology, 19(6), 629-633.

26. Adhikari, H. S., & Yadav, P. N. (2018). Anticancer activity of chitosan, chitosan derivatives, and their mechanism of action. International Journal of Biomaterials, 2018.

27. Punarvasu, T. P., & Prashanth, K. H. (2022). Self-assembled chitosan derived microparticles inhibit tumor angiogenesis and induce apoptosis in Ehrlich-ascites-tumor bearing mice. Carbohydrate Polymers, 278, 118941.

28. Jiang, Z., Han, B., Li, H., Li, X., Yang, Y., & Liu, W. (2015). Preparation and anti-tumor metastasis of carboxymethyl chitosan. Carbohydrate polymers, 125, 53-60.

29. Edis, Z., Wang, J., Waqas, M. K., Ijaz, M., & Ijaz, M. (2021). Nanocarriers-mediated drug delivery systems for anticancer agents: an overview and perspectives. International journal of nanomedicine, 1313-1330.

30. Soleimanbeigi, M., Dousti, F., Hassanzadeh, F., Mirian, M., Varshosaz, J., Kasesaz, Y., & Rostami, M. (2021). Boron phenyl alanine targeted chitosan–PNIPAAm core–shell thermo-responsive nanoparticles: boosting drug delivery to glioblastoma in BNCT. Drug Development and Industrial Pharmacy, 47(10), 1607-1623.

31. Ryu, J. H., Yoon, H. Y., Sun, I. C., Kwon, I. C., & Kim, K. (2020). Tumor-targeting glycol chitosan nanoparticles for cancer heterogeneity. Advanced Materials, 32(51), 2002197.

32. Amin, M. Z., Afrin, M., Meghla, N. S., Nur, M. A., Rahman, M. M., & Uddin, M. J. (2021). Assessment of antibacterial, anti-inflammatory, and cytotoxic effects of different extracts of Gynura procumbens leaf. Current Therapeutic Research, 95, 100636.

33. Idris, S. A., Markom, M., Abd Rahman, N., & Ali, J. M. (2019, November). Quantitative HPLC analysis of flavonoid in three different solvent extracts in leaves of Gynura procumbens. In Journal of Physics: Conference Series (Vol. 1349, No. 1, p. 012003). IOP Publishing.

34. Sutthammikorn, N., Supajatura, V., Yue, H., Takahashi, M., Chansakaow, S., Nakano, N., ... & Niyonsaba, F. (2021). Topical Gynura procumbens as a novel therapeutic improves wound healing in diabetic mice. Plants, 10(6), 1122.

35. Ketuly, K. A. (2011). Acute toxicity study and wound healing potential of Gynura procumbens leaf extract in rats. Journal of Medicinal Plants Research, 5(12), 2551-2558.

36. Timotius, K. H., & Rahayu, I. (2021). Ethnopharmacological relevance of Eusideroxylon zwageri Teijsm. et Binnend: A review. Seed, 51, 23.

37. Khatun, M., Nur, M. A., Biswas, S., Khan, M., & Amin, M. Z. (2021). Assessment of the anti-oxidant, anti-inflammatory and anti-bacterial activities of different types of

turmeric (Curcuma longa) powder in Bangladesh. Journal of Agriculture and Food Research, 6, 100201.

38. Cao, M. Y., Wu, J., Xie, C. Q., Wu, L., Gu, Z., Hu, J. W., & Xiong, W. (2022). Antioxidant and anti-inflammatory activities of Gynura procumbens flowers extract through suppressing LPS-induced MAPK/NF-κB signalling pathways. Food and Agricultural Immunology, 33(1), 511-529.

39. Samraj, S., & Rajamurgugan, S. (2017). Qualitative and quantitative estimation of bioactive compounds and antioxidant activity in Citrus hystrix. International Journal of Engineering Science and Computing, 7(6), 13154-13163.

40. Wulandari, L., Siswanti, R. A. Y. N., & Nugraha, A. S. (2019). Determination of total phenolic content and classification model of local variety soursop (Annona muricata L.) leaf powder in different altitudes using NIR and FTIR spectroscopy coupled with chemometrics. J. Pharm, 30(1), 7-14.

41. Wulandari, L., Siswanti, R. A. Y. N., & Nugraha, A. S. (2019). Determination of total phenolic content and classification model of local variety soursop (Annona muricata L.) leaf powder in different altitudes using NIR and FTIR spectroscopy coupled with chemometrics. J. Pharm, 30(1), 7-14.

42. Akhi, T. M. N., Adib, M., Islam, Q. S., Sultana, I., Haider, R., & Ibrahim, M. (2019). Preliminary Phytochemical Screening and Assessment of Pharmacological Activities of Leaves and Stems of Gynura procumbens (Lour.) Merr. Bangladesh Pharmaceutical Journal, 22(1), 79-84.

43. Kaewseejan, N., Sutthikhum, V., & Siriamornpun, S. (2015). Potential of Gynura procumbens leaves as source of flavonoid-enriched fractions with enhanced antioxidant capacity. Journal of functional foods, 12, 120-128.

44. Chakraborty, D., & Shah, B. (2011). Antimicrobial, antioxidative and antihemolytic activity of Piper betel leaf extracts. International Journal of Pharmacy and Pharmaceutical Sciences, 3(3), 192-199.

45. Ranzato, E. (2019). Scratch Wound Healing Assay. *Methods in Molecular Biology*, 225–229. https://doi.org/10.1007/7651_2019_259

46. Kaewseejan, N., Sutthikhum, V., & Siriamornpun, S. (2015, January). Potential of Gynura procumbens leaves as source of flavonoid-enriched fractions with enhanced antioxidant capacity. *Journal of Functional Foods*, *12*, 120–128. https://doi.org/10.1016/j.jff.2014.11.001

47. Mitra S, Gaur U, Ghosh PC, Mitra AN. Tumor targeted delivery of encapsulated dextran-doxorubicin conjugate using chitosan nanoparticles as carrier. J Control Release. 2001; 74:317–323.

48. Park J, Fong PM, Lu J, et al. PEGylated PLGA nanoparticles for the improved delivery of doxorubicin. Nanomedicine. 2009; 5:410–418.

49. Krasnici S, Werner A, Eichhorn ME, et al. Effect of the surface charge of liposomes on their uptake by angiogenic tumor vessels. Int J Cancer. 2003; 105:561–567

50. Clogston, J. D., & Patri, A. K. (2010, October 19). Zeta Potential Measurement. *Methods in Molecular Biology*, 63–70. https://doi.org/10.1007/978-1-60327-198-1_6

51. Sporn, M. B., & Suh, N. (2002, July). Chemoprevention: an essential approach to controlling cancer. Nature Reviews Cancer, 2(7), 537–543. https://doi.org/10.1038/nrc844

52. Yeong, L. T., Abdul Hamid, R., Saiful Yazan, L., Khaza'ai, H., & Mohtarrudin, N. (2015, December). Low dose triterpene-quinone fraction from Ardisia crispa root precludes chemical-induced mouse skin tumor promotion. BMC Complementary and Alternative Medicine, 15(1). https://doi.org/10.1186/s12906-015-0954-3

53. Nordin, M. L., Abdul Kadir, A., Zakaria, Z. A., Abdullah, R., & Abdullah, M. N. H. (2018, March 12). In vitro investigation of cytotoxic and antioxidative activities of Ardisia crispa against breast cancer cell lines, MCF-7 and MDA-MB-231. BMC Complementary and Alternative Medicine, 18(1). https://doi.org/10.1186/s12906-018-2153-5

54. Amita Pandey, Shalini Tripathi. Concept of standardization, extraction and pre phytochemical screening strategies for herbal drug. J Pharmacogn Phytochem 2014;2(5):115-119.

55. Kolodziej, H., & Kiderlen, A. F. (2005, September). Antileishmanial activity and immune modulatory effects of tannins and related compounds on Leishmania parasitised RAW 264.7 cells. Phytochemistry, 66(17), 2056–2071. https://doi.org/10.1016/j.phytochem.2005.01.01

56. Baharum Z, Akim AM, Taufiq-Yap YH, Hamid RA, Kasran R. In vitro antioxidant and antiproliferative activities of methanolic plant part extracts of Theobroma cacao. Molecules. 2014;19(11):18317–31.

57. Atjanasuppat K, Wongkham W, Meepowpan P, Kittakoop P, Sobhon P, Bartlett A, Whitfield PJ. In vitro screening for anthelmintic and antitumour activity of ethnomedicinal plants from Thailand. J Ethnopharmacol. 2009;123(3):475–82.

58. Lee CC, Houghton P. Cytotoxicity of plants from Malaysia and Thailand used traditionally to treat cancer. J Ethnopharmacol. 2005;100(3):237–43.

59. Boik J. Natural compounds in cancer therapy. Minnesota: Oregon Medical Press; 2001.

60. Zoe, L. H., David, S. R., & Rajabalaya, R. (2023, December). Chitosan nanoparticle toxicity: A comprehensive literature review of in vivo and in vitro assessments for medical applications. Toxicology Reports, 11, 83–106. https://doi.org/10.1016/j.toxrep.2023.06.012

61. Zoe, L. H., David, S. R., & Rajabalaya, R. (2023, December). Chitosan nanoparticle toxicity: A comprehensive literature review of in vivo and in vitro assessments for medical applications. *Toxicology Reports*, *11*, 83–106. https://doi.org/10.1016/j.toxrep.2023.06.012

62. Mohammed, M., Syeda, J., Wasan, K., & Wasan, E. (2017, November 20). An Overview of Chitosan Nanoparticles and Its Application in Non-Parenteral Drug Delivery. *Pharmaceutics*, *9*(4), 53. https://doi.org/10.3390/pharmaceutics9040053

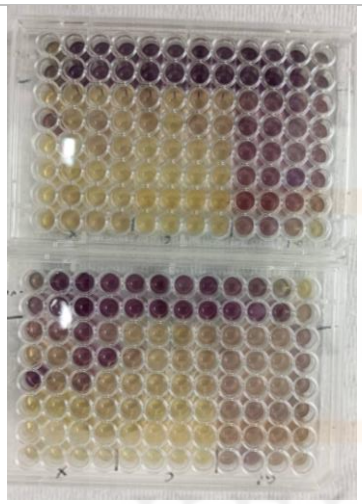
13.0 Appendices

Appendix A: shows the chitosan with medium molecular weight.

Appendix B: shows the process of sonication

Appendix C: shows the Particle size analyser

Appendix D: Shows the process of plant extraction using Soxhlet apparatus



Appendix E: Shows the 96 well plates that was used for cell cytotoxicity assay

Appendix F: show the result of Gynura Procumbens extraction after filtration