FORMULATION OF THYMOQUINONE-LOADED PLGA NANOPARTICLES AND CYTOTOXICITY IN MALIGNANT MELANOMA A375 CELL LINE

BY

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ABSTRACT

Background: Thymoquinone (TQ) has been known to have various therapeutic benefits but it is prone to fast degradation. TQ exhibited anticancer effect in several cancer cell lines. Encapsulation of TQ enhances its stability and provides a tool for targeting to the cancer tissues. **Objective:** To prepare and optimize TQ- poly-lactide-co-glycolide (PLGA) nanoparticle (NP) formulation and evaluate it in A375 cells for cell uptake and cytotoxicity. **Methodology:** TQ- PLGA NP formulation was prepared using solvent evaporation method and TQ quantification method was validated using HPLC. The total of 6 factors affecting the formulation (chitosan concentration (A), ratio of co-stabilizer (tween 80: polyvinyl alcohol (B), stabilizer concentration (C), ratio of co-solvents (ethyl acetate: dichloromethane) (D), sonication power (E) and sonication time (F), were screened using fractional factorial design. The screened factors were reduced to 3 most significant (A, C and E) affecting critical output responses of particle size, zeta potential and encapsulation efficiency. An optimized TQ-PLGA NP was prepared by altering these significant factors using full factorial design and targeted responses was acquired and it was proceed for cell uptake studies by flow cytometer and fluorescence microscope; and cell cytotoxicity studies by MTT assay. **Results and discussion:** Optimized TQ-PLGA NP formulation was prepared using 1.02 % w/v chitosan, 1.14% w/v stabilizer and 31% of sonication power. The rest of the parameters were fixed as 1:3 oil to aqueous phase ratio, 1:5 primary emulsion to dispersion medium ratio, 3% w/v PLGA concentration and 75:25 co-solvent ratios of ethyl acetate and dichloromethane. TQ-PLGA NP exhibited particle size of 147.2 ± 0.4 nm; polydispersity index (PDI) of 0.142 ± 0.017; zeta potential of 22.1 ± 1.1; encapsulation efficiency of 96.81 ± 0.05 %; biphasic release of 56.7 ± 1.3% (24 h) and 69.7 ± 1.3% within 1 week. The optimized formulation was aggregated in powdered form but stable in the suspension form up for 10 days. The formulation exhibited lower glass transition of PLGA below 37°C suggesting a plasticizing effect of TQ and other ingredients which contributes to the overall rapid release. TQ in methanol and TQ-PLGA NP in complete growth media were tested for stability. TQ degraded by 18% (24 h) and 77.0% (48 h), particle size remains stable in 24 h, significant changes occurred after 48 h ($p < 0.05$), PDI changes were significant post 24 h and 48 h ($p < 0.05$), significant changes on zeta potential were observed on higher concentration of TQ-PLGA NPs ($p < 0.05$). The highest intracellular uptake was at 1.0 mg/mL NP concentration with time-dependent cell uptake up to 24 h. Post 24 h treatment, the IC50 of TQ-PLGA NP and TQ-solution was calculated to be 4.428 mg/mL and 91.71 µg/mL respectively. At 48 h treatment, IC50s of TQ-PLGA NP was calculated to be 7.981 mg/mL and 58.657 mg/mL respectively. **Conclusion:** Optimized TQ-PLGA NP was formulated and it showed a promising cytotoxic effect in A375 cells. TQ in nanoparticle formulation has a potential use as anticancer and worth a further study in animal models.

خلاصة البحث

الخلفية: من المعروف أن الثيموكينون (TQ) له فوائد علاجية مختلفة ولكنه عرضة للتدهور السريع. أظهر TQ تأثيرا مضادا للسرطان في العديد من خطوط الخلايا السرطانية. يعزز كبسلة TQ من استقراره ويوفر أداة لاستهداف الأنسجة السرطانية. **الهدف:** إعداد وتحسين تركيبة الجسيمات النانوية (NP) المؤلفة من TQ مع بولي-لاكتايد-كو-جليكولايد (PLGA) وتقييمها في خلايا A375 بناء على الامتصاص الخلوي والسمية الخلوية. **المنهجية:** تم تحضير تركيبة جسيمات PLGA-TQ النانوية باستخدام طريقة تبخير المذيبات وتم التحقق من طريقة قياس TQ الكمية باستخدام HPLC. باستخدام التصميم الجزئي للعوامل تم فحص 6 عوامل مؤثرة على التركيبة: (أ) تركيز الشيتوزان، (ب) نسبة المثبت الثانوية (توين 80: كحول البولي فينيل، (ج) تركيز المثبت، (د) نسبة المذيبات الثانوية (إيثيل الأسيتات:ثنائي كلورو الميثان)، (هـ) قوة الصوتنة، (و) ومدة الصوتنة. وتم تصفية العوامل التي تم فحصها إلى الثلاثة الأكثر أهمية (أ، ب، ج) والتي تؤثر على استجابات النواتج الحرجة لكل من حجم الجسيمات، وكمون زيتا، وكفاءة الكبسلة. تم تحضير جسيمات TQ-PLGA النانوية عن طريق تغيير هذه العوامل الهامة باستخدام التصميم العاملي الجزئي وقد تم الحصول على الاستجابات المستهدفة والتقدم لمرحلة دراسات امتصاص الخلايا بواسطة مقياس التدفق الخلوي والمجهر التألقي؛ ودراسات السمية الخلوية بواسطة فحص MTT. **النتائج والمناقشة:** تم تحضير جسيمات TQ-PLGA النانوية المحسنة باستخدام 1.02٪ w/v شيتوسان، و 1.14٪ w/v مثبت، و 31٪ من قوة الصوتنة. تم تثبيت بقية المؤشرات كالتالي: 1:3 نسبة المرحلة الزيتية إلى المرحلة المائية، 1:5 نسبة المستحلب الأولي إلى وسط التشتت، 3٪ w/v تركيز PLGA، و 75:25 نسب المذيب الثانوي لأسيتات إيثيل وثاني كلورو الميثان. أظهرت جسيمات TQ-PLGA النانوية حجم 147.2±0.4 نانومتر؛ ومؤشر تشتت متعدد 0.142±0.017 ؛ وكمون زيتا 22.1±1.1 ؛ وكفاءة كبسلة بنسبة 96.81±0.05٪ ؛ وإطلاق ثنائي الطور 56.7±1.3٪ خلال 24 ساعة و 69.7±1.3٪ خلال أسبوع واحد. تم تجميع التركيبة المحسنة على شكل مسحوق ولكنها كانت مستقرة على شكل مستعلق لمدة 10 أيام. أظهرت الصيغة تحولا زجاجيًا منخفضًا لـ PLGA بدرجة حرارة أقل من 37 درجة مئوية مما يشير إلى التأثير التلديني لـ TQ والمكونات الأخرى مما ساهم في الإطلاق السريع بشكل عام. تم اختبار استقرار الـ TQ في الميثانول و جسيمات TQ-PLGA النانوية في وسائط استزراع كاملة، وأظهرت النتائج تدهور الـ TQ بنسبة 18٪ (في 24 ساعة) و بنسبة 77.0٪ (في 48 ساعة)، وظل حجم الجسيمات ثابتًا خلال 24 ساعة ولكن ظهرت تغيرات ملحوظة بعد 48 ساعة ($p<0.05$)، وكانت تغيرات مؤشر التشتت المتعدد ملحوظة بعد 24 ساعة و 48 ساعة ($p<0.05$)، ولوحظت تغيرات ملحوظة في كمون زيتا على التراكيز العالية لجسيمات TQ-PLGA النانوية ($p<0.05$). كان أعلى امتصاص داخل الخلايا عند تركيز 1.0 مجم/مل من الجسيمات النانوية مع امتصاص خلوي معتمد على الوقت حتى 24 ساعة. بعد المعالجة لمدة 24 ساعة، تم حساب الـ IC_{50} حيث بلغ 4.428 مجم/مل لجسيمات TQ-PLGA النانوية و 91.71 ميكروجرام/مل لـ TQ-مذيب. عند المعالجة لمدة 48 ساعة تم حساب IC_{50} لجسيمات TQ-PLGA النانوية وبلغت 7.981 مجم/مل و 58.657 مجم/مل على التوالي. **الخلاصة:** تمت صياغة جسيمات TQ-PLGA النانوية المحسنة وأظهر سمية خلوية واعدة في خلايا A375. لدى TQ في صياغة الجسيمات النانوية استخدام واعد كمضاد للسرطان ويستحق دراسة إضافية في النماذج الحيوانية.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master in Pharmaceutical Sciences (Pharmaceutical Technology).

……..……
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Supervisor

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Luqman Muizzuddin Bin Mohd Rosli

Signature ..... Date .....

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Firstly, it is my utmost pleasure to dedicate this work to my dear parents and my family, who granted me the gift of their unwavering belief in my ability to accomplish this goal: thank you for your support and patience.

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LIST OF ABBREVIATIONS

A375	Melanoma skin cancer cells
BRAF	BRAF is a human gene that encodes a protein called B-Raf
C6-PLGA NP	Coumarin-loaded Polylactic-Glycolic acid nanoparticles
CS	Chitosan
DAPI	4′,6-diamidino-2-phenylindole (fluoresecent stain)
D1	Day 1
D2	Day 2
DSC	differential scanning calorimetry
EE	encapsulation efficiency
EPR	enhanced permeability and retention
FTIR	Fourier – Transform Infrared Spectroscopy
HPLC	High Performance Liquid Chromatography
ICH guideline	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guideline
ICH Q2	Validation of Analytical Procedures: Text and Methodology
LOD	Limit of detection
LOQ	Limit of quantification
MEK	Mitogen-activated protein kinase kinase enzymes MEK1 and/or MEK2
MSC	Melanoma skin cancer
NMSC	Non-melanoma skin cancer
PBS	phosphate buffer saline
PDI	Polydispersity index
PLGA	Polylactic-glycolid acid
PS	Particle size
PVA	Polyvinyl alcohol
RSD	Relative standard deviation
SEM	Scanning Electron Microscopy
T80	Tween 80
TQ	Thymoquinone
TQ-PLGA NP	Thymoquinone-loaded Polylactic-Glycolic acid nanoparticles
TrypLE E	TrypLE express enzyme
UV	Ultraviolet
UV light	Ultraviolet light
ZP	zeta potential

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Malignant melanoma of cell line A375 is a type of cancer develops from pigment containing cells called melanocytes and being featured as one of the top invasive of its kind, it has low prognostic value (Hofmann et. al, 2000). The metastatic type of its tumor development stage is among topmost aggressive and drug resistance. Patient's median survival time is about 5 – 6 years and has the survival rate less than 5% (Lens & Dawes, 2004).

Ahmad et. al, (2013) reported in his study the pharmacological properties of *Nigella Sativa* were attributes to quinine derivative called Thymoquinone (TQ) which present as most abundant active constituent (30% - 48%) in it as compared to other active compound like thymohydroquinone, dithymoquinone, p-cymene (7%-15%), carvacrol (6%-12%), 4-terpineol (2%-7%), t-anethol (1%-4%), sesquiterpene longifolene (1%-8%). TQ has been known to have therapeutic effect such as antimicrobial, antioxidants, hepatoprotective, anti-inflammatory and anticancer effects. It is believed that the mechanisms involved are inhibition of cell proliferation and eliciting apoptosis and necrosis mainly through interference of transcription factors, growth factors, inflammatory cytokines, protein kinases and other enzymes (Banerjee et. al, 2010). Nanotechnology in drug carrier system has skyrocketed in recent years which have led to more advanced treatment and ease diagnosis of many diseases including cancers.

Historically, the use of TQ came from revelation, the essence of Black cumin; the black seed oil (*Nigella Sativa*) has undergone tremendous studies for ages. The

black seed is one of the famous traditional medicines that were prescribed by prophet Muhammad (PBUHAHF) as narrated that the Prophet said: “this black seed is a healing for all diseases except Al-Sam.’ I said, ‘What is Al-Sam?’ He said: ‘Death’.” Narrated by Al-Bukhari (1976).

In couple of decades ago, PLGA has been of the most interesting polymer to be fabricated in application for drug delivery. PLGA is known for its biocompatibility and biodegradability and Food and Drug Administration (FDA) has approved its application for drug delivery and tissue engineering. This has resulted in extensive studies of PLGA development for controlled delivery of drugs, proteins and other macromolecules.

This study aims to develop a stable nanoparticle formulation for TQ and evaluate its cytotoxic effect in A375 cell line.

1.2 STATEMENT OF THE PROBLEM

Chemotherapeutic agents have many serious side effects such as risk of infections, risk of bleeding and prone to fatigue apart from other minor side effects (Gunjal et. Al., 2015). In the interest of these setbacks, TQ as natural product may offer as safer alternative to the chemotherapeutic drugs in treating melanoma skin cancer (Reddy, Odhav, & Bhoola, 2003).Yet an issue arises from the instability of TQ if administered alone as it degrades fast in aqueous environment (Salmani et. al, 2014). In case in parenteral route of administration, TQ may not reach insufficient concentration to the cancer site due to fast degradation and lack of targeting mechanism. Hence, the role of drug carrier is to encapsulate the TQ thus increase the stability in aqueous environment hence increasing therapeutic efficacy for treating melanoma. In addition,

it provides a tool for accumulating the drug in the cancer site due to the leaky nature of the cancer blood vessels.

Among other benefits of nano-drug delivery system besides provide stability in harsh environment are biodegradability, biocompatibility, high encapsulation characteristics, and probability of surface modification (Patravale, Dandekar, & Jain, 2012). The most important issue that lead to this study is TQ formulated in PLGA nanoparticles have yet to be studied in melanoma skin cancers and this study aim to test this dosage form in a stable formulation of TQ against the melanoma skin cancer cell lines.

1.3 RESEARCH OBJECTIVE

1.3.1 General Objective

To prepare and evaluate stable formulation of TQ-loaded PLGA nanoparticles (TQ_PLGA NP) through optimization of the formulation (particle size, zeta potential and efficiency) and the cell uptake and cytotoxicity.

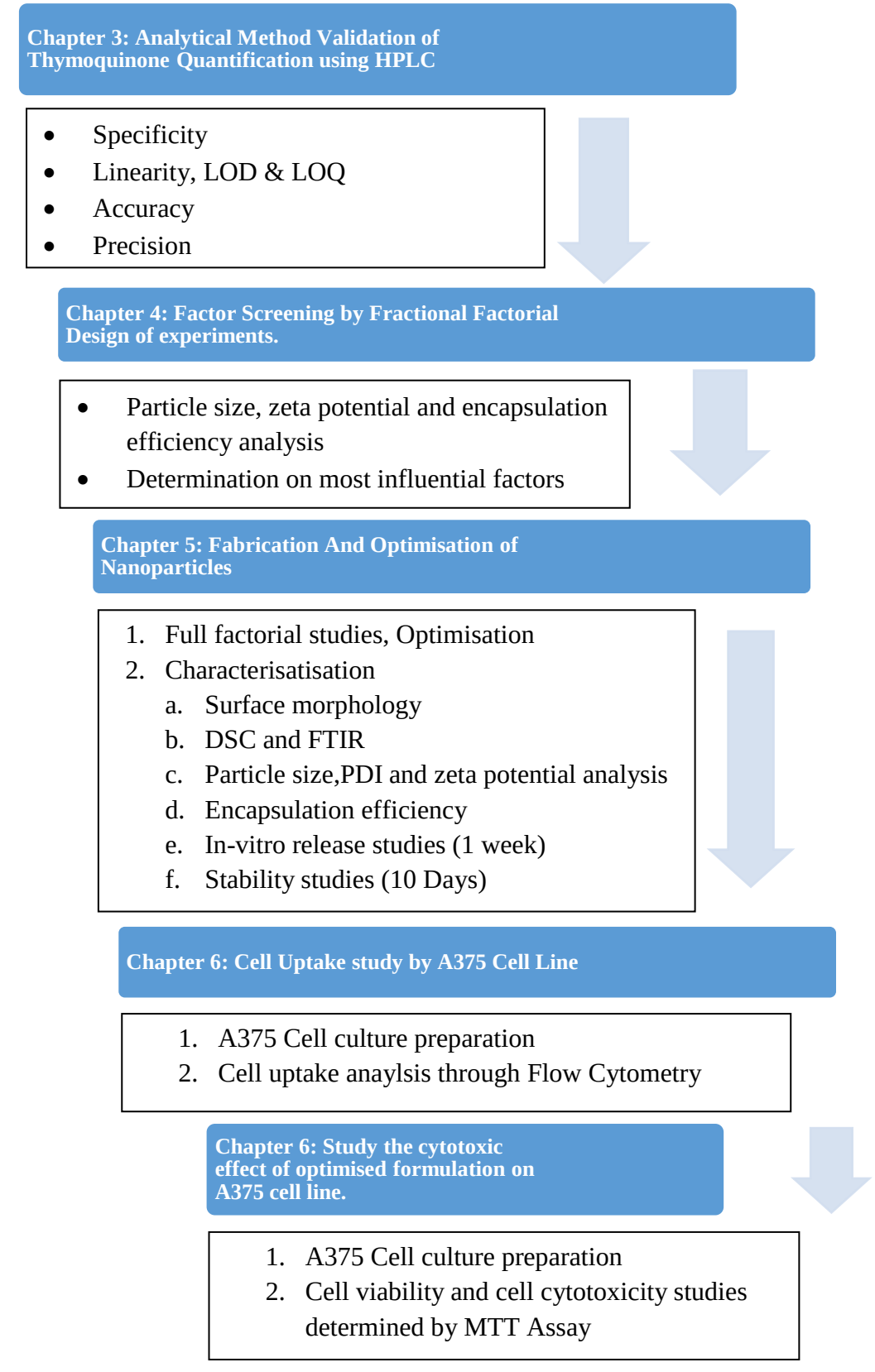
1.3.2 Specific Objective

1) To validate an analytical method of quantifying TQ using HPLC.
2) To screen influencing factors affecting the formulation using fractional factorial design.
3) To formulate and optimize TQ-PLGA NP through full factorial design.
4) To evaluate the cell uptake of C6-PLGA NP and TQ-PLGA NP by A375 cell line
5) To study the cytotoxic effect of the optimized formulation on the cell line.

1.4 RESEARCH QUESTIONS

1. Can stable formulation of TQ-PLGA NP be formulated?
2. Will there be significant cell uptake of TQ-PLGA NP by malignant melanoma A375 cell line?
3. Will TQ-PLGA NP show cytotoxic effect on A375 cell line?

1.5 FLOW OF THE STUDY



CHAPTER TWO

LITERATURE REVIEW

2.1 MALIGNANT MELANOMA SKIN CANCER

Malignant melanoma is a cancerous skin disease of melanocytes; cells located on the lower part of epidermis and responsible for melanin secretion, the pigment of skin Figure 2.1 shows the anatomy of human skin. In normal physiologic condition, melanocytes create melanin and cause darker skin under ultraviolet (UV) exposure. Melanoma can be initiated anywhere on the skin surface and even though melanoma cases are uncommon, melanoma tends to initiate tissue invasion and spread to other body parts when it becomes cancerous. Solar UV radiation exposure, age, fair complexion, history of skin cancer, genetic factors or family history of melanoma malignancies, infection with human papilloma virus, weak immune system, organ transplantation and use of tanning beds are some risk factors that have been linked to melanoma (America Cancer Society, 2013; Gordon, 2013). The visual presentation of malignant melanoma is shown in Figure 2.2.

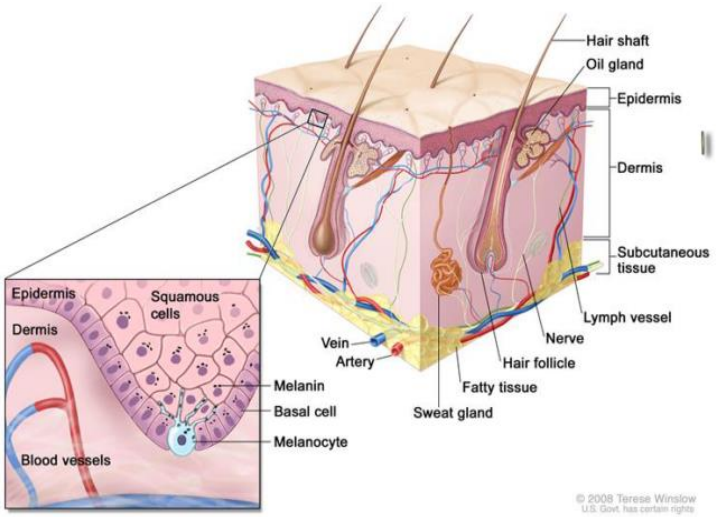


Figure 2.1 Normal anatomy of the skin
Source: The National Cancer Institute, available at
https://www.cancer.gov/types/skin/patient/melanoma-treatment-pdq

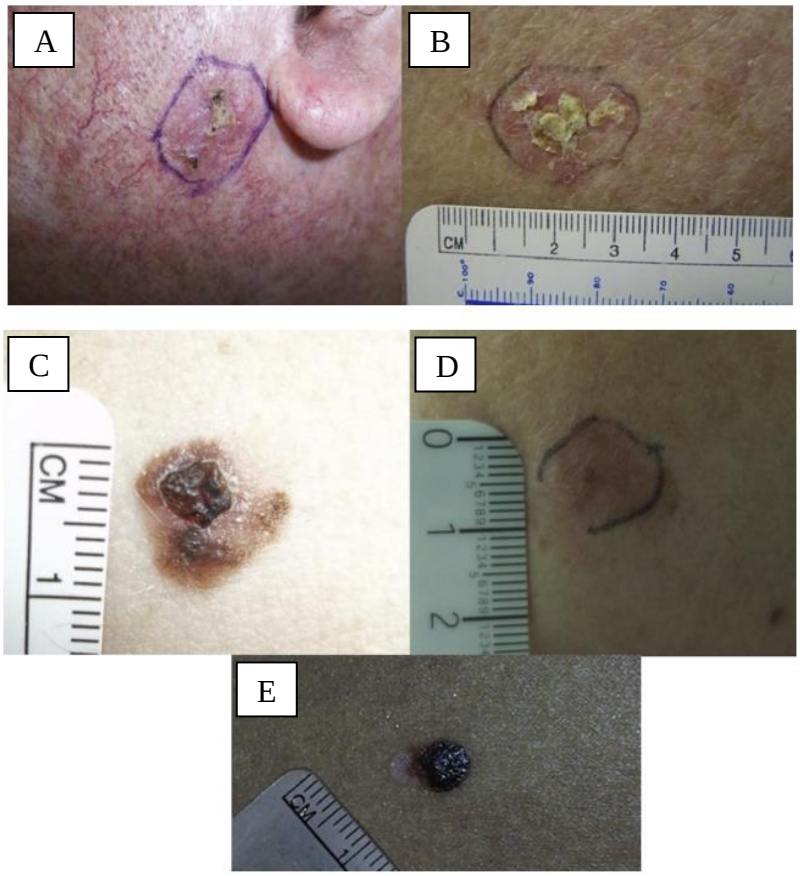


Figure 2.2 A, B, C, D, E shows representation of malignant melanoma of the skin.
(Adapted from Gordon, 2013)