Development of biodegradable sustained-release damnacanthal nanocapsules for potential application in *in-vitro* breast cancer studies

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Abstract: The "noni" species of Morinda citrifolia L., is using in traditional medicine in the tropical country for over 2000 years. Noni fruit has come from the Morinda citrifolia tree which is called Rubiaceae, and it is from the coffee family. It is a perennial herb whose ripe fruit has a robust butyric acid smell and flavor. Recently scientists have proven that this fruit has antioxidant and antibiotic properties in vitro. An anthraquinone, damnacanthal, is one of the constituents of Morinda citrifolia. It has been demonstrated to have anti-cancer properties. Damnacanthal has low water solubility and low bioavailability. Formulating of damnacanthal into the biodegradable nanocapsule drug delivery system may increase its bioavailability. Various formulations of damnacanthal would be developed to enable the selection of a dosage form that could offer the provision of the anti-cancer bioactive substance with suitable sustained- or controlled release properties. The efficiency of extraction of damnacanthal will be compared using both conventional and traditional method. Both the damnacanthal and an anthraquinone active compounds extracted from noni roots, are currently being studied in the context of anti-cancer study. Soon, the medical values, bioactivities and nutritional of this fruit can be assessed, especially its anti-cancer activity, this fruit extract could play an outstanding economic role in Malaysia and other tropical countries.

Keyword: Damnacanthal, subcritical water extraction, nanocapsules, drug delivery, PLGA, anti-cancer.

INTRODUCTION

Problem statement

Morinda citrifolia is known in Malaysia as mengkudu or noni. It is one of the traditional folk medicinal plants in Polynesia and Hawaii for over 2000 years due to its beneficial health effects. In botany, this species is called Apatot. Apatot is an erect, small tree, or smooth herb, 2.5 to 13 meter high. Leaves are sketchily elliptic to rhombus, 10 to 24 cms lengthy, with faced and rounded tips. Peduncles are leaf-opposed, lonely, 1.5 to 2.5 cms lengthy. Flowers have form dense and, not collaborated, heads rounded, and are 1.5 to 2.5 cms. Corolla is white and Calyx is truncate, 1 cm long; limb is 5-lobed, 1 cm in diameter. Fleshy fruit, greenish white in fig. 1, ovoid, 3 to 10 cms lengthy, with the fragrance of decaying cheese Above 140 phytochemical bioactive compounds have been traced from this fruit, including damnacanthal. Damnacanthal is from the anthraquinone family of aromatic organic compounds and it is chemically written as (3-hydroxyl-1-methoxy-anthraquinone-2- aldehyde), initially isolated from the roots of noni, it is a type of anthraquinones with potential anti-cancer and anti-tumor effects. Many studies had shown its anti-cancer activity including inhibiting the formation of tumors or by apoptosis increasing in cell lines of cancer. Though, clinical applications of damnacanthal for the treatment of cell lines of cancer and many chronic diseases have been generally stuck by its lipophilic property, in which it has poor water solubility, resulting in poor bioavailability. Therefore, nanotechnology-based drug delivery system is proposed as a proper delivery system to enhance the efficacy of feebly soluble drugs for systemic release.

Breast cancer is known as a significant global health problem and the leading cause of death among women. National Cancer Registry of Malaysia provides an age-standardized incident rate (ASR) of 46.2 per 100,000 in 2014, which means 1 in 2000 women in Malaysia may be diagnosed with breast cancer. Breast cancers, as with other most tumors, are treated with a combination chemo- and adjuvant therapies. However, these treatments have been associated with severe side effects and sometimes are life-threatening. The application of nanotechnology to medical science may offer a potential and better solution to the historical challenge that has adjudicated breast cancer so trying to contain and eradicate.

Damnacanthal could be extracted from the roots and flesh of noni fruits via conventional and non-conventional extraction methods and purified. Purified damnacanthal will be loaded in PE-Gylated-PLGA poly (lactic-co-glycolic acid) nanocapsules for the therapy of cell lines of

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cancer breast cancer. According to literature search, so far no study has done the therapeutic potential of damnacanthal-nanocapsules for the treatment of breast cancer *in vitro*.

Fig. 1: Morinda citrifolia fruit

Literature reviews

Researchers and medical professionals have been investigating Morinda citrifolia plant for many centuries as a potential natural resource for various therapeutic effects. Morinda citrifolia, also commercially known as Noni plant, is a small tropical tree and found in open coastal and forest areas up to 1300 feet above sea love. M. citrifolia belongs to the Rubiaceae family and is frequently used in Hawaiian and Polynesian old-fashioned remedies. In other countries, M. citrifolia is also known as Nuna in India, mengkudu in Malaysia, nhaut in Southeast Asia, ba Ji tian in China and cheese fruit in Australia. It has been reported that M citrifolia has a broad range of health benefits, including anti-fungal, antiviral, analgesic, anti-inflammatory, anti-cancer and anti-tumor activities.

Fig. 2: Morinda citrifolia tree leaf

More than 180 phytochemical bioactive compounds have been identified and isolated from different parts of M. citrifolia, including fruit in fig. 1, a leaf in fig. 2, bark and root in fig. 3, with its significant micronutrients being alkaloids and phenolic compounds. The chemical compositions and their concentrations are related significantly not only to the plant parts but also to its harvesting seasons and country of origin.

According to D. Krishnaiah et al. their book on medicinal plants Malaysian researchers have found, Morinda citrifolia chemical elements are: 5,6- dihydroxylucidin, ajmalicine isomers, alizarin, asperuloside, chrysophanol (1,8-dihy droxy-3-methylanthraquinone), mono-ethoxyrubiadin, morindadiol, morindin, a-methoxyali-zarin, 5,7-Acacetin-7-O-bD(+)-glycopyranoside, asperu-losidic acid, damnacanthal, digoxin, 5,6-dihydroxylu-cidin-3-b-primeveroside, 5,7-dimethylapigenin- 40 -O-b-D(+)-galacto pyranoside, lucidin, lucidin-3-b-primeveroside, 2-methyl-3,5,6-trihydroxy anthraquinone, 3-hydroxymorindone, 3-hydroxymorindone-6 -b-primereroside, 2-methyl-3,5,6-trihydrox yanthraquinone-6-b-primeveroside, morindone (1,5,6-trihydroxy-2-methylanthraquinone), morindone-6-b-primeveroside, nordamnacanthal, quinoline, rubiadin, rubiadin 1-methyl ether, saronjidiol, ursolic acid, alkaloids, anthraquinones and their glycosides, glucose (b-Dglucopyranose), caprylic acid, caproic acid, fatty acids and alcohols (C5-9), flavonoid, flavones glycosides, s,b-sitosterol and indoles, purines.

Fig. 3: Morinda citrifolia tree root

Among all the constituents extracted from M. citrifolia, the most important compounds that have potential anti-cancer activity are damnacanthal. Damnacanthal or chemically known as 3-hydroxy-1-methoxy-anthraquinone-2-aldehyde is a type of anthraquinone and appears as pale-yellow crystals with the melting point of 210-211ºC. Damnacanthal has initially extracted from the aromatic phenolic phase of M. citrifolia root extracts. However, it can also be found in other parts of the plant. Damnacanthal has been identified as a potent inhibitor of p56lck tyrosine kinase activity, a protein activity embraced in the chemotactic response of T cells to CXCL12, through high-volume screening of natural products. It has been shown to prevent other tyrosine kinases, that's are insulin receptor, EGFR and erbB2. Besides, damnacanthal also exhibits cytotoxic activity against various types of cancer cells, including breast cancer and small cell lung cancer cell lines. Another study by Alitheen et al. showed that damnacanthal have successfully exhibited to be cytotoxic towards various types of leukemia cell lines without causing toxicity towards normal cell 3T3.

Even though damnacanthal has attracted considerable benefits due to the possibility of its anticancer potential, however, damncanthal for the treatment of cancer, clinical

applications and other chronic disease have been prevent due to damncanthal poor solubility in aqueous, which is crucial in determining the capability of the compound to cross the cell membranes, resulting in reduced bioavailability. The development of an arterial nanodrugs based formulation of damnacanthal for the treatment of cancer is needed.

Nano drugs carriers have been of significant interest to many medicinal scientists over the last decade as they offer many great benefits as drug delivery systems as they can overcome the limitations found in conventional cancer therapy. In spite of their particularly limited range of minute sizes (1-100 nm), nano drugs carriers could be made using a variety of materials including biodegradable polymers and biodegradable polymeric nanoparticles, dendrimers), nanoparticles by lipids, solid-lipid, liposomes, magnetic nanoparticles and carbon nanotubes, virus-like particles and biological materials and protein cage nanoparticles.

The use of nanocarriers in various biomedical applications has also been known for their ability to alter drug's pharmacokinetics. For example, nanocarriers can improve the solubility of poorly soluble drugs and reduce metabolism by dissolving them in their hydrophobic or hydrophilic compartments. Several liposomal-nanocarrier formulations have been developed for this purpose. Zhang et al. has developed a formulation encapsulating by lyophilized liposomal a chemotherapeutic drug called paclitaxel. This formulation was able to achieve high drug loading for doses up to $0.325gmm^{-2}$ which are much higher than the recommended dose of $0.175gmm^{-2}$. Another example of a nanocarrier used to deliver poorly soluble drugs was demonstrated by Yang et al. in which camptothecin, an anti-neoplastic chemotherapy drug, was able to achieve a higher therapeutic efficiency after oral administration when encapsulated in solid lipid nanoparticles. Besides, nanocarriers can be modified not just to carry drugs, but also carry imaging probes. Imaging probes in nanoparticles are commonly used to not only localize a tumour but also to confirm the status of cancer itself, including if cancer has metastasized to any part of the other organs. Cohen et al. has made a formula near-infrared fluorescence (NIRF) albumin nanoparticles using near-infrared dye derivative and human serum albumin, which is used for in vivo detection of colon cancer. Another researcher, Moore et al. has developed NIFR Cy5.5 dye-labeled iron oxide drugs, which is used as both magnetic resonance (MR) and NIFR-imaging contrast to gathering information on tumor localization, environment, and status.

In this study, we propose using biodegradable polymeric nanocapsules as a nano drugs carrier. Polymeric nanocapsules can achieve high drug loading in water-insoluble, facilitate intra-tumoural distribution and protect the active agent from any premature dilapidation while providing the drug as sustained- or controlled-release delivery. Encapsulated nanoparticles exhibit in a core-shell structure it allowing any active molecules to keep to the oil core that is walled by a polymer molecules as a coating

Two different methods can produce Nano capsules: template-based and self-assembly. In the template-based methodology, a polymer shell can be surrounded beside a particle as a template that can finally be eliminated and then leaving an empty polymeric shell frame can be grouped in aqueous solution into vesicular frame and the hollow sphere morphology of these aggregates twisted allowing them as a suitable precursor for the preparation of stable nanocapsules inventions. Alternatively, core size nano particles can be synthesized by oil in water (o/w/e) emulsion polymerization, followed by the addition of a distinctive and singular monomer that results in the formation of a cross-linked sphere morphology beside a core particle, followed by the subtraction of the core particles.

M. citrifolia natural components anti-cancer activity are mostly reported as a spontaneous anticancer cure where sulphated polysaccharide stops metastasis by destabilizing the interaction between glycosaminoglycan and specific proteins while damnacanthal inhibits the formation of tumors either by interfering with the growth of ras gene activation, and by increasing apoptosis in human colorectal cancer cell lines. Alizarin has an antiangiogenic effect on blocking blood circulation to malignant cancer cells. Liver, and lung cancers prevents with limonene mammary, by stimulating thymus gland to secrete more T. cells which destroy the carcinoma cells. Ursolic acid inhibits the growth of cancerous cells and induces apoptosis by modulating the immune body process. Thai M. citrifolia fresh and dried leaf dichloromethane extracts were reported to be more efficient and probably safer in treating cancer than M. citrifolia pure compounds such as damnacanthal, rutin, and scopoletin due to the extracts higher safety ratios. For human cancer cell lines (cervical carcinoma, breast carcinoma, hepatocellular carcinoma, and epidermoid carcinoma) and a Vero (kidney of African green monkey) cell lines were used in the study. Both M. citrifolia extracts showed an inhibitory effect on cervical carcinoma cells and epidermoid carcinoma, while the pure compounds, rutin, and scopoletin, showed lower anti-proliferative effects on all human cancer cell lines. However, only damnacanthal have effective cytotoxic effects against all human cancer cell lines as well as African green monkey kidney cell lines

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Malaysian government policy

The Malaysia government through "The Development of Agro-based SMEs through Technology Transfer from Government Research Institution" policy is promoting the

small and medium enterprises (SMEs) to be involved in agricultural entrepreneurship. Noni fruit cultivation will be encouraged among the local SMEs and the findings from this research would not just lead to an increased use of Noni fruit but will also boost the country's economic growth.

The Ministry of Science, Technology and Innovation (MOSTI) has established a National Nanotechnology Directorate (NND) which provides as the national focal point for the coordination of research and development related to nanotechnologies. This research project will contribute to the country's development in nanotechnology, specifically in the health science sector. In this work optimise the extraction and isolation methodology of damnacanthal from the flesh of noni fruits, encapsulate bioactive compound damnacanthal in PEGylated PLGA nanocapsules and will try to achieve an in vitro therapeutic effects of damnacanthal-loaded nanocapsules in breast cancer cell lines.

Possible extraction and partitioned sequentially, using ethyl acetate and n-butanol (3×20) as a solvent. The ethyl acetate fraction (300 g) will be go through a column of silica gel (72×12 cm), using n-hexane and ethyl acetate mixtures with improving polarity as an eluent. All fractions (Fr) collected as follows: fraction-1 [n-hexane, 4000mL), Fr-2 [nhexane-ethyl acetate (49:1), 3000mL], Fr-3 [n-hexane-ethyl acetate (45:5), 4000mL], Fr-4 [n-hexane-ethyl acetate (37:13), 4000mL], Fr-5 [n-hexane-ethyl acetate (40:10), 4000mL], Fr-6 [n-hexane-ethyl acetate (35:15), 3000mL], Fr.-7 [n-hexane-ethyl acetate (33:17), 3000mL], Fr-8 [nhexane-ethyl acetate (30:20), 4000mL], Fr.-9 [n-hexaneethyl acetate (28:22), 3000mL], Fr-10 [n-hexane-ethyl acetate (25:25), 3000mL], Fr-11 [n-hexane-ethyl acetate (24:26), 3000mL], Fr-12 [, n-hexane-ethyl acetate (22:28), 4000mL], Fr-13 [nhexaneethyl acetate (20:30), 3000mL], Fr-14 [n-hexane-ethyl acetate (17:33), 3000mL], Fr-15 [n-hexane-ethyl acetate (15:35), 4000mL], Fr-16 [n-hexane-ethyl acetate (12:38), 4000mL], Fr-17 [n-hexaneethyl acetate (10:40), 4000mL], Fr.-18 [n-hexane-ethyl acetate (5:45), 4000mL], Fr.-19 [n-hexane-ethyl acetate (1:49), 3000mL], and Fr.-20 (ethyl acetate, 6000mL). Further purification of the Fr.-12 will be performed on column of silica gel (from ethyl acetate: dichloromethane = 1:200 to 100% ethyl acetate) to give damnacanthal. Nuclear magnetic resonance spectra will be obtained in CDCl3 (deuterated chloroform) at a fixed temperature controlled and regulated to around 300 K on a Varian Mercury plus 400 NMR spectrometer, and the residual proton resonance (chloroform) of deuterated chloroform will be used as an internal shift of reference. The 2D NMR spectra will be recorded by using standard pulse cycles. EIMS (Electron impact mass spectrometry) will be recorded on Finnigan TSQ- 700 mass spectrometer. TLC (Thin-layer chromatography) will be performed by using 60 F254 plates of silica gel (Merck). Column chromatography will be conduct on (230–400 mesh) silica gel (ASTM, Merck). HPLC (High-performance liquid chromatography) will be used on a (Hitachi L- 2130) apparatus equipped with a (Hitachi L-2455) photodiode array detector. A Lichrosorb silica gel 60 (5μm) column (250 × 10 mm) will be used for semi-preparative HPLC separation.

Extraction and isolation of high yield damnacanthal from Morinda citrifolia fruits

I. Damnacanthal-loaded PLGA-PEG nanocapsules formulation,
II. In vitro therapeutic effects of Damnacanthal-loaded PLGA-PEG nanocapsules against breast cancer cell lines

M. citrifolia fruit cultivation, nano-technology industry, and future breast cancer treatment will be motivated by this research.

Isolation of damnacanthal

Sample preparation

Fresh samples of Malaysian Morinda citrifolia will be collected from local suppliers and then separated to fruit parts (flesh and seed). The seed will be removed, and the flesh part will be continuosly washed with water for eliminating of superfluous impurity. After washing finished, sample will be frozen immediately at -80ºC and then lyophilized at -40ºC. The sample flesh will be ground to make a mould by an automatic grinder and subsequently sieved to get <1 mm particles. Flesh sample stored in an air tight container in a dry place at 16-20ºC, until use. Standard damnacanthal (>99% purity) will be purchased from supplier as a positive control. Damnacanthal will be extracted and purified using two methods; solvent extraction or subcritical water extraction method.

Solvent extraction method of damnacanthal

M. citrifolia (32kg) of Lyphophilised powder will be extracted with methanol (3×80 L) at room temperature (7 days each). The methanol extract will be evaporated in vacuum to afford a residue, which was suspended in Dw (deionized water) (3 L) and then partitioned sequentially, using ethyl acetate and n-butanol (3×2 l) as solvent. The ethyl acetate fraction (300 g) will be passed through a column of silica gel (72×12 cm), using solvent of n-hexane and ethyl acetate mixtures with improving polarity as an eluent.

DISCUSSION

Anthropological cells are frequently exposed to reactive oxygen radicals generated by some biotic and abiotic factors such as stress, environmental factors, irradiation, pollutants, and by-products of metabolic processes. When the exposure overwhelms endogenous preventive systems, cells are exposed to a potentially harmful load of

oxidants, leading to various free radicals induced pernicious effects. Free radical attacks biological molecules such as enzymes, proteins, lipids, DNA and RNA leading to cell or tissue injury associated with many diseases including carcinogenesis aging, heart diseases, and atherosclerosis, etc. Antioxidants are compounds which act as radical scavengers when added to the food products and prevent the extreme chain reaction of oxidation, inhibit or delay the oxidation process and increase shelf life by retarding the means of lipid peroxidation. Thus the antioxidant activities of the combined extract and the eight individual isolated compounds were studied. The SFE extract showed the highest antioxidant activity of 95% at 800µ g/mL. The high antioxidant potential for the extract may be due to the combined effect of the individual phytochemicals present in the extract.

The antioxidant activities of the individual compounds decreased in the order: damnacanthal >β-sistosterol >campesta-5-22- trien-3-ol>stigmasteroid > ergosteroid > stigmasta-4-en-3-one >E-phytol>stigmasta-4-22-dien-3-one. Between the isolated multipart damnacanthal showed the high antioxidant activity when compared to other compounds, and for this reason, damnacanthal was considered for further studies. During the past decades, the killing of tumours through the induction of apoptosis has been recognized as a novel strategy for the identification of anticancer drugs.

Apoptosis originally referred to an active form of cell death with stereotypic morphological physiognomies happening during the development. A wide range of pathological conditions can induce apoptosis. Unbalanced cell explosion and apoptosis may play a role in the pathogenesis of certain types of tumors and neurodegenerative diseases. Our study showed that the damnacanthal proved the most active cytotoxic (Induction of apoptosis) effect on HepG2 (78%) at 24 h when compared to extract whose IC_{50} value is µ 132µg/mL. damnacanthal had a much smaller IC_{50} value (54µg/mL) as compared to that of hexane extract, suggesting the former is more effective against HepG2 cell proliferation than the latter, and they were compared with standard cyclophosphamide (95.3%). Caspases present in mitochondria are the crucial mediators of apoptosis. Of the 14 caspases identified in mammals, caspase-3, previously called CPP32, Yama, apopain is the major downstream protease in all apoptotic pathways. The most notorious apoptogenic factor released from permeabilized mitochondria is the respiratory component cytochrome c, which recruits apoptosis protease activating factor called Apaf-1 and procaspase-9 to form apoptosome, caspase-9 is thus enabled, and orchestrates caspase-3 and other effector molecules for the cell death. From our study, it was evident that, when damnacanthal was added to the culture medium, a significant increase in the caspases -3 and caspases-9 protein levels were observed. Moreover, the dose-dependent up-regulation of caspases-3 and caspases-9 activation by damnacanthal was confirmed. The p53 pathway is preferentially used control the apoptosis machinery. Roy et al. reported that epigallocatechin-3- gallate inhibited HepG2 cell proliferation and induced apoptosis via p53-dependent and Fas-mediated pathways. Alkaloids are main bioactive chemicals in nux vomica, and they are effective against different types of cancer. The present study indicates that damnacanthal one of the isolated compound from the hexane extract up-regulates caspase-3 expression, which leads to an enhancement in apoptosis susceptibility. We also demonstrated here for the first time that the potentiation of caspase-3 expression by damnacanthal is mediated via the p53-dependent pathway. The results indicate that damnacanthal is the primary bioactive compound from the hexane extract and supports the further research and development of the bioactive ingredients from M. pubescens leaves as anticancer agents, especially against liver cancer.

CONCLUSION

In this review we can be concluded the following findings mostly through in conventional methods; fruit contains physiochemical: flavoring, aligns, saccharides, irids, nonresident, completion, catechist and catecholamine, neanderthal, alkaloids. Studies have yielded completion, vitamin C, steroids, alkaloids, octogenarian acid, potassium, anthracites, ergosterol, e-carotene, vitamin A, Lavonne triglycerides and Oleanolic acid. Fruit juice composition of noni juice ingredients per g/100g juice(J) and dry matter in juice(DM) yields: water 90.25(J) 0 (DM); lipids 0.15 (J), 1.5±0.05 (DM); proteins 2.5 (J), 25.6±0.3 (DM); mineral matter 0.86 (J), 8.8±0.3 (DM); fibres 3.38 (J), 34.7±0.1 (DM); total sugars 2.01 (J), 20.6 ±0.2 (DM); chlorophyll 0.03 (J), 0.30±0.004 (DM); mineral and organic anions 0.82 (J), 8.4±0.1 (DM);. Leaves yield flavourful triglycerides, beta-carotene, and iridium triglycerides. A general study of methanol extracts of fruits, stems, and leaves yielded almost 22 ingredients. Eight were new compounds: morindicinone, phenolphthalein, morinthone, moribundity, naphthalene, molindone, 5-benzofuran carboxylic acid -6-formyl methyl ester, and morindicininone with 14 known components viz., 1, 3-dimethoxyanthraquinone, hydroquinone, scopoletin, 2,4-dimethoxy-9-anthrone, 1, 8-dihydroxy-6- methoxy-3- methyl-9- anthrone, 2-hydroxymethyl anthraquinone, 2-hydroxyanthraquinone, 2-methoxy anthraquinone, 1-hydroxy-2-methylanthraquinone, stearic acid, palmitic acid, and 4-hydroxy-4-[1'E,3'R)-3'-hydroxyl'butenyl]-3,5,5'-trimethyl-cyclohexane-2-en-l-one and 1.2- dihydroxyanthraquinone, 4-(3' (R)-hydroxybutyl) -3,5,5'-trimethyl –cyclohexane -2-en-l-one. Ethanolic extract of fruit juice and leaves yielded fifteen and eighteen compounds respectively.

Among them were n-decanoic acid, octanoic acid, hexanoic acid, allantoin, sorbitol, cyclopropyl, gamma tocopherol, and glycerin mannitol. Fifty-one volatile constituents have been identified, including organic acids such as hexanoic acids and octanoic, alcohols including 3-methyl-3-butene-1-ol, and esters like methyl octanoate, and methyl decanoate, as ketones 2-heptanone, and lactones (E)-6-dodecenoic-y-lactone. Root bark contains a moraine ($C_{27}H_{10}O_{15}$), crystal glucose, and a coloring matter, moraine. A few *in vitro* and *in vivo* animal studies recommend a possible discovered substance in unpasteurized noni fruit, leaf and roots that may have a of anticancer activity. Active component isolation justifies further research. Our reviews results suggest that encapsulated damnacanthal exhibits better activity in cell growth inhibition, compared to non-encapsulated damnacanthal. So, damnacanthal has potential to be a product for the development of chemoprevention or therapeutic agents for cancers.

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