

ANTI-CANCER ACTIVITY OF RUTIN ENCAPSULATED IN LOW METHOXYL PECTIN BEADS

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Received: 12 Feb 2014, Revised and Accepted: 28 Apr 2014

ABSTRACT

Objective: This study was investigating the anti-cancer activity of rutin encapsulated in non-amidated low methoxyl pectin bead in various formulations with or without sorbitol and/or sodium bicarbonate (NaHCO₃).

Methods: The rutin beads formulations including 3NA (3% Non-amidated LMP), 3NA15Sor (3% Non-amidated LMP with 15% Sorbitol), 3NA1Bica (3% Non-amidated LMP with 1% NaHCO₃) and 3NA15Sor1Bica (3% Non-amidated LMP with 15% Sorbitol and 1% NaHCO₃) were prepared and the *in vitro* anti-cancer activities in 3 human cancer cell lines were investigated.

Results: All beads were oblong or spherical shape with around 600 µm size. 3NA15Sor1Bica rutin bead composing both sorbitol and NaHCO₃ exhibited higher cytotoxic activity with cancer cell viability were 51.41 ± 7.01 and 55.12 ± 3.96% on HT-29 and KB cells, respectively. There was no significant different (*p*>0.05) of cell viability between all bead formulation for HepG2 cells.

Conclusion: These results confirmed that encapsulation of rutin in pectin beads exhibited their anti-cancer activity and can be applied for further human cancer treatment formulation.

Keywords: Low methoxyl pectin; Rutin; Anti-cancer activity; Beads.

INTRODUCTION

Chemotherapy is essential for the treatment of a variety of cancers. Cancer which is characterized by the lack of the control cell proliferation and differentiation that can invade organs and tissues is a major public health burden both in developed and developing countries. Many anti-cancer agents from natural sources including plants, marine organisms and micro-organisms were tested for cytotoxic activity against cancer in cell lines grown *in vitro* or *in vivo* animal cancer models. Many flavonoids-containing medicinal plants have antitumor property such as flavonoids from licorice extract may be useful chemopreventive agents for peptic ulcer or gastric cancer in *Helicobacter pylori* infected individuals [1]. Sophorane which was extracted from a traditional Chinese medicine (Shan Dou Gen), inhibited cell growth and induced apoptosis in various lines of cancer cells such as human stomach cancer (MKN7) cells and human leukemia (U937) cells [2]. Rutin (quercetin-3-*O*-rutinoside), the flavonol glycoside of quercetin, is abundantly found and distributed in plants such as buckwheat seed, fruits and fruit rinds, especially citrus fruits and berries. It presents important properties in human health like its significant scavenging properties on oxidizing species such as hydroxyl radical, superoxide radical and peroxy radical [3]. Numerous studies have shown that rutin has anti-cancer effects. Lin et al (2009) reported that rutin induced cell cycle arrest and apoptosis in murine leukemia (WEHI-3) cells *in vitro* and *in vivo* [4].

Many researchers have been spent their time in the development of new anti-cancer drugs to improve clinical outcomes with minimal toxicity. Some are developing the optimal drug delivery system to overcome the issues of systemic toxicity as well as to improve the efficiency and stability of drug or active compounds. A number of studies have focused on the design of carriers for delivery to provide optimal incorporation of drugs [5-7]. Microencapsulation has developed significantly and is applied in various fields such as pharmaceuticals, cosmetics and food. This allows for controlled release of various drugs and enhanced stability of formulations [8]. Moreover, microencapsulation provides a useful technique to protect active molecules from environmental conditions (e.g. sensitivity to air/oxygen, heat and light, enzyme, acidic pH in stomach), to extend shelf-life [9, 10]. Thus, rutin which is the light sensitive compound could be encapsulated in order to enhance its stability. However, there are no data showing about the activities of

encapsulated rutin on human cancer cells. In this present study, rutin encapsulated in non-amidated low methoxyl pectin bead in various formulations with or without sorbitol and/or sodium bicarbonate were prepared as well as *in vitro* anti-cancer activities in 3 human cancer cell lines including human colon adenocarcinoma (HT-29) cells, human mouth epidermal carcinoma (KB) cells and human hepatocellular carcinoma (HepG2) cells were investigated in order to evaluate their potential for further human cancer treatment formulation.

MATERIALS AND METHODS

Materials

Non-amidated low methoxy pectin (LMP) (Unipeptine OF300C; DE = 30% and DA = 0%) were purchased from Cargill™ (Saint Germain, France). Rutin hydrate, sodium bicarbonate (NaHCO₃), sulforhodamine B, tris (hydroxymethyl)-methlamine and trichloroacetic acid (TCA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were obtained from GIBCO (Grand Island, NY, USA). Sorbitol pure anhydrate was purchased from Cooperation Pharmaceutique Francaise (Melun, France). Hydrochloric acid (HCl) 1N and sodium hydroxide (NaOH) 1N were purchased from Prolabo® (Fontenay-sous-bois, France). All reagents were analytical grade.

Preparation of low methoxyl pectin (LMP) beads by encapsulator

The ionotropic gelation technique of using drug encapsulated in the beads [11-13] was modified as following: LMP aqueous formulations including 3NA (3% Non-amidated LMP), 3NA15Sor (3% Non-amidated LMP with 15% sorbitol), 3NA1Bica (3% Non-amidated LMP with 1% NaHCO₃) and 3NA15Sor1Bica (3% Non-amidated LMP with 15% sorbitol and 1% NaHCO₃) were prepared followed by 2% w/v of rutin dispersed in the solution and stirred until a uniform dispersion was obtained. The pH (SevenEasy™, pH meter S20, Schwerzenbach, Switzerland) and viscosity (HAAKE Viscotester 550, Thermo Scientific, France) of the solution were measured. The beads were made using Encapsulator UNIT VAR1 (Nisco engineering Inc., Zurich, Switzerland) with a nozzle of 0.7 mm inner diameter. The slurry was dropped into 50 ml of a gently agitated solution of the

crosslinking agent (2% w/v CaCl_2) at flow rate 100 mL/h with falling distance of 4 cm. The gelled beads were formed immediately and allowed to stand in the cross-linking solution for 10 min. Then beads were separated by filtration, washed with deionized water and dried at $37 \pm 2^\circ\text{C}$ for 24 h in a drying room.

Morphological studied

Morphological examination of the LMP beads were conducted by scanning electron microscopy (SEM) using a JEOL scanning electron microscope (JSM-6400F) at 10 kV. LMP beads were coated with nickel solution, under vacuum by SPI Sputter coating unit. The experiments were performed at magnifications $\times 75$. Size and shape of beads were evaluated [14].

Cell culture

Human colon adenocarcinoma (HT-29), human mouth epidermal carcinoma (KB) and hepatocellular carcinoma (HepG2) cells were cultured under the standard conditions in the complete culture medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 mg/mL). Cells were incubated in a temperature-controlled, humidified incubator (Shel Lab, model 2123TC, USA) with 5% CO_2 at 37°C .

Cytotoxic activity by the Sulforhodamine B (SRB) assay

Rutin and rutin encapsulated in non-amidated low methoxyl pectin bead with or without sorbitol and/or sodium bicarbonate at five serial concentration as well as the standard anti-cancer drugs (Cisplatin and 5-Fluorouracil at the concentration ranging from 0.1 to 1000 $\mu\text{g}/\text{mL}$) were tested for cytotoxic activity on 3 human cancer cell lines by the SRB assay according to the method of Papazisis et al [15]. Briefly, the cells were plated at the density of 1.0×10^4 cells/well in 96-well plates and left for cell attachment on the plate for 24 h in 5% CO_2 at 37°C . Cells were then exposed to five serial concentrations of the beads and non-encapsulated rutin in complete culture medium (0.01-0.5 mg/mL) for 24 h. After incubation, the cells were fixed with 50% trichloroacetic acid solution incubated at 4°C for 1 h and washed five times with distilled water. The excess water was drained off and the plates were air-dried for 24 h. The cells were stained with 50 μL of 0.4% SRB solution in 1% acetic acid for 30 min at room temperature ($25 \pm 2^\circ\text{C}$).

After incubation, the SRB solution was poured off and the plates were washed with 1% acetic acid; until only the cell adhered dye was left. The plates were air-dried and 100 μL of 10 mM Tris-base solution was added to each well to solubilize the dye. The mixture was shaken for 30 min at room temperature ($25 \pm 2^\circ\text{C}$) and the absorbance was measured at 540 nm by a Microplate Reader (Biorad model 680, Biorad, Japan). The experiment was performed in triplicate. The percentages of cell viability were calculated using the following equation: % cell viability = $(A_{\text{sample}} - A_{\text{blank}} / A_{\text{control}} - A_{\text{blank}}) \times 100$, whereas A_{sample} was the optical density of the cells treated with the sample, A_{control} was the optical density of the non-treated cells and A_{blank} was the optical density of Tris-base solution at time zero. The IC_{50} values, the concentrations inhibit 50% of cell viability were obtained by plotting the percentages of cell viability versus the concentrations of the sample.

Statistical analysis

The results were presented as the mean of three independent experiments and the standard deviation (SD). ANOVA was used for the analysis of the test results at the significance level of p -value < 0.05 .

RESULTS AND DISCUSSION

Beads preparation and morphological studied

When the pectin droplets came in contact with the calcium chloride solution, ionic interaction occurred and formed gelled spheres. These interactions in non-amidated LMP allowed the formation of a compact pectin network, in which rutin particles were encapsulated.

All beads were oblong or spherical shape with around 600 μm size. Scanning electron micrographs of the beads are presented in Figure 1. The surface of the sphere of 3NA and 3NA15Sor rutin-loaded beads showed a rough and globular morphology (Figure 1A and 1B), while 3NA1Bica and 3NA15Sor1Bica rutin-loaded beads presented the smoother surface (Figure 1C and 1D). Some researchers found that added NaHCO_3 in the polymer matrix could be attributed to the presence of entrapped gas bubbles increasing porosity of beads [16-18] but it was not clearly indicated in this study and that may be due to the lower NaHCO_3 concentration used in 3NA15Sor1Bica and 3NA1Bica formulations.

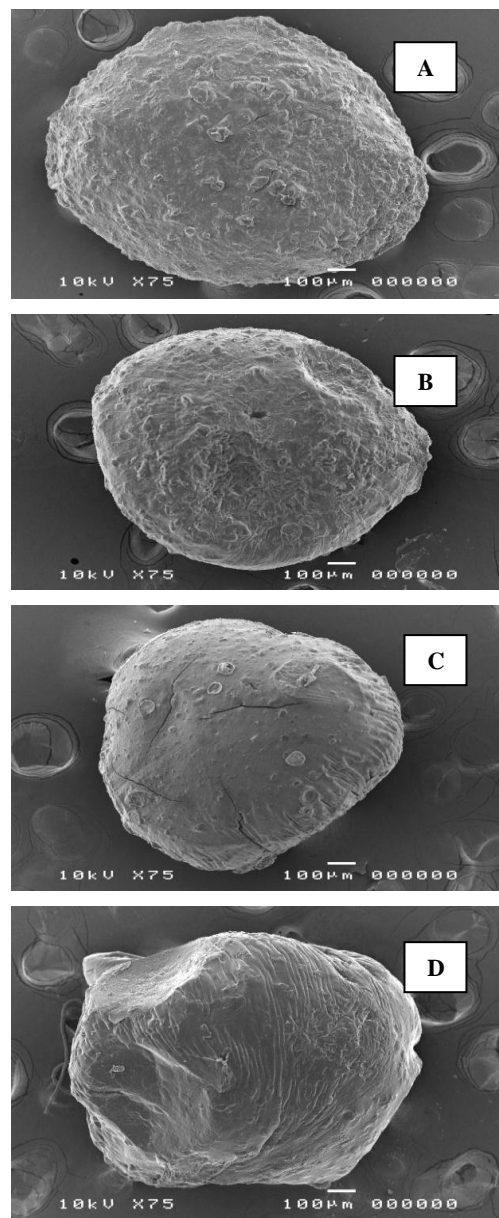


Fig. 1: Scanning electron microscope (SEM) morphology of 3NA (A), 3NA15Sor (B), 3NA1Bica (C) and 3NA15Sor1Bica (D)

Cytotoxic activity on human cancer cell lines

The cytotoxicity (% cell viability) of the 4 formulations of low methoxyl pectin beads and standard anticancer drugs (Cisplatin and 5-Fluorouracil) on 3 human cancer cell lines were shown in Tables 1 and 2.

Table 1: The percentages of cell viability (%) at the highest non-encapsulated and encapsulated rutin concentration (0.5 mg/ml) on three cancer cell lines

Formulations	% Cell viability		
	HT-29	KB	HepG2
3NA	73.57 ± 12.82	77.76 ± 4.64	65.37 ± 2.82
3NA15Sor	76.79 ± 6.09	74.87 ± 3.97	62.54 ± 7.69
3NA1Bica	62.86 ± 7.29	65.15 ± 6.11	61.41 ± 3.25
3NA15Sor1Bica	51.41 ± 7.01	55.12 ± 3.96	62.98 ± 7.66
Non-encapsulated rutin	89.70 ± 2.64	81.39 ± 2.13	87.11 ± 7.53

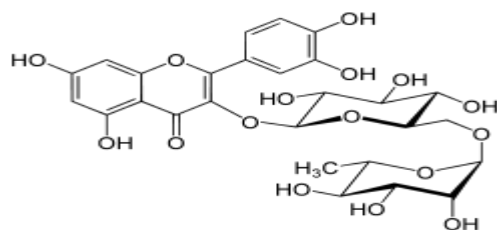
Notes: 3NA = 3% Non-amidated LMP, 3NA15Sor = 3% Non-amidated LMP with 15% Sorbitol, 3NA1Bica = 3% Non-amidated LMP with 1% NaHCO₃ and 3NA15Sor1Bica = 3% Non-amidated LMP with 15% Sorbitol and 1% NaHCO₃ with 2% w/v of rutin; human colon adenocarcinoma (HT-29) cells, human mouth epidermal carcinoma (KB) cells and human hepatocellular carcinoma (HepG2) cells

Table 2: Cytotoxic activity (IC₅₀) on three cancer cell lines of standard anticancer drugs

Standard anticancer drugs	IC ₅₀ (µg/ml)		
	HT-29	KB	HepG2
Cisplatin	1.10 ± 0.33	4.51 ± 0.66	26.40 ± 3.37
5-Fluorouracil	155.18 ± 3.37	12.94 ± 2.19	203.18 ± 5.51

Notes: human colon adenocarcinoma (HT-29) cells, human mouth epidermal carcinoma (KB) cells and human hepatocellular carcinoma (HepG2) cells

Rutin, a glycoside of quercetin, has been shown to have anti-mutagenic action. It is considered to be both an inhibitor of carcinogenic processes and a potential cancer chemo preventive agent [19-21]. In 2004, Undeğer et al investigated the modulating effects of the two flavonoids quercetin and rutin on the mutagenic anticancer drug Mitomycin C by single cell gel electrophoresis in human lymphocytes. In human lymphocytes quercetin and rutin displayed protective effects on DNA damage induced by Mitomycin C in a concentration-dependent manner [22]. In addition, Volate et al (2005) compared the ability of four herbal flavonoids (quercetin, curcumin, rutin, and silymarin) and one whole herb mixture (ginseng powder) to suppress aberrant crypt foci in an azoxymethane-induced rat colon cancer model. The results of their study suggested that these herbal supplements may exert significant and potentially beneficial effects on decreasing the amount of precancerous lesions and inducing apoptosis in the large intestine [23]. However, rutin is a poorly water-soluble (12.5 mg/100 mL of water) compound, despite the fact that it has 10 hydroxyl groups (four phenolic hydroxyl groups and six sugar hydroxyl groups, Figure 2). Therefore, non-encapsulated rutin did not completely dissolved in culture medium which composed of water as the major compartment. Rutin (non-encapsulated one), which was dissolved in poor solvent (culture medium) exhibited very low anti-cancer activity (cell viability more than 80%) in this study.

**Fig. 2: Chemical structure of rutin**

In all bead formulations, the rutin beads composing with NaHCO₃ (3NA15Sor1Bica and 3NA1Bica) revealed lower cancer cells viability which were 51.41 ± 7.01 and 62.86 ± 7.29% in HT-29 cells and 55.12 ± 3.96 and 65.15 ± 6.11% in KB cells, respectively. 3NA15Sor1Bica rutin bead formulation which composing both sorbitol and NaHCO₃ exhibited the significant ($p < 0.05$) lowest the percentages of cancer cell viability on HT-29 and KB cells when comparing in all bead formulations (Table 2). This result probably dues to NaHCO₃ in the formulations having dissolved and increased the pH of culture medium that may increase the diffusion and solubility of rutin into

the cancer cells. In fact, rutin is freely soluble in alkaline solutions [24-26], and adding the sorbitol did not change the pH of the medium solution. In addition, pectin and sorbitol molecules could compete for cations. Depending on the sorbitol structure a stable complex can be formed between sorbitol and Ca²⁺. This interaction can be unfavorable to the gel formation, due to the decrease of Ca²⁺ available to associate with pectin molecules and therefore the decreasing of the rigidity [27]. For HepG2 cells, there was no significant different between all bead formulation ($p > 0.05$). Standard anticancer drugs which were Cisplatin and 5-Fluorouracil showed better anti-cancer activity with IC₅₀ in microgram range (Table 2). The using of standard anticancer drugs as positive control confirms that the basic conditions of the experiment were exhibited positive results and also good practice of our replicate samples of the testing.

CONCLUSION

This present study has demonstrated the *in vitro* anti-cancer activities of various formulations of rutin encapsulated in low methoxyl pectin beads. The rutin which was encapsulated in 3NA15Sor1Bica (3% Non-amidated LMP with 15% sorbitol and 1% NaHCO₃) bead showed anti-cancer activity higher than non-encapsulated rutin on human cancer cell lines especially on human colon adenocarcinoma (HT-29) and human mouth epidermal carcinoma (KB) cells. The rutin encapsulated in beads have not been previous studied for anti-cancer activity and further assesse the molecular mechanism should be evaluated.

ACKNOWLEDGEMENT

This work was supported by Faculty of Pharmacy and Chiang Mai University. The authors sincerely thank Prof. Dr. Aranya Manosroi, mentor, under the New Researcher Project 2013, Chiang Mai University, Thailand.

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