

Antiproliferation, antioxidation and induction of apoptosis by *Garcinia mangostana* (mangosteen) on SKBR3 human breast cancer cell line

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Abstract

This study was designed to determine the antiproliferative, apoptotic and antioxidative properties of crude methanolic extract (CME) from the pericarp of *Garcinia mangostana* (family Guttiferae) using human breast cancer (SKBR3) cell line as a model system. SKBR3 cells were cultured in the presence of CME at various concentrations (0–50 µg/ml) for 48 h and the percentage of cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-di phenyl tetrazolium bromide (MTT) assay. CME showed a dose-dependent inhibition of cell proliferation with ED₅₀ of 9.25 ± 0.64 µg/ml. We found that antiproliferative effect of CME was associated with apoptosis on breast cancer cell line by determinations of morphological changes and oligonucleosomal DNA fragments. In addition, CME at various concentrations and incubation times were also found to inhibit ROS production. These investigations suggested that the methanolic extract from the pericarp of *Garcinia mangostana* had strong antiproliferation, potent antioxidation and induction of apoptosis. Thus, it indicates that this substance can show different activities and has potential for cancer chemoprevention which were dose dependent as well as exposure time dependent.

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1. Introduction

Breast carcinoma (BC) is the commonest cancer among women and the second highest cause of cancer death (Merrill and Weed, 2001). Most cases occur during age 45–55. It also occurs in men but is more than 100-fold less frequent than in women (Cooper, 1992). At present, the cancer treatment by chemotherapeutic agents, surgery and radiation have not been fully effective against the high incidence or low survival rate of most the cancers. The development of new therapeutic approach to breast cancer remains one of the most challenging area in cancer research.

Many tropical plants have interesting biological activities with potential therapeutic applications. *Garcinia mangostana* Linn (GM), family Guttiferae, is named 'the

queen of fruits' because many people agree that it is one of the best tasting fruit in the world. It can be cultivated in the tropical rainforest such as Indonesia, Malaysia, Philippines and Thailand. People in these countries have used GM (mangosteen) as traditional medicines for the treatment of abdominal pain, diarrhoea, astringent, dysentery, infected wound, suppuration, chronic ulcer, leucorrhoea and gonorrhoea (Satyavati et al., 1976). Moreover, the studies revealed that GM has anti-inflammatory (Gopalakrishnan et al., 1980), antitumour, antioxidant (Williams et al., 1995) and antibacterial activities on *Staphylococcus aureus*, *Shigella dysenteriae*, *Shigella flexneri*, *Escherichia coli*, *Vibrio cholerae* (Farnsworth and Bunyapraphatsara, 1992) and *Helicobacter pylori* (Mahabusarakum et al., 1983). The pericarp (peel) of GM was reported to be the source of mangostin, tannin, xanthone, chrysanthemine, gartanin, Vitamin B1, B2, C and other bioactive substances (Farnsworth and Bunyapraphatsara, 1992).

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From the above traditional usages and later scientific findings suggested that the GM is a potential candidate as an anticancer agent. It is very likely that the traditional uses especially in the treatment of abdominal pain, leucorrhoea and chronic ulcer are related to the anti-inflammatory and antioxidant properties of GM. Although many benefits of GM have been claimed, only few authentic scientific studies are available. The present investigation was undertaken to evaluate the antiproliferation, apoptosis and antioxidant of crude methanolic extract (CME) from GM using SKBR3 human breast cancer cell line as a model.

2. Materials and methods

2.1. Reagents

RPMI 1640 medium and foetal calf serum (FCS) were obtained from Biochrom (Berlin, Germany). Hanks' balanced salt solution (HBSS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), propidium iodide (PI), Benzimidazole Hoechst 33342 (Ho33342), 2',7'-dichlorodihydro fluorescein diacetate (DCFH-DA) and α -tocopherol (Vitamin E) were purchased from Sigma (St. Louis, MO). Proteinase K was purchased from Promega (Madison, WI) and RNase A was from Amresco (Buckinghamshire, UK).

2.2. Plant material

GM were purchased from fresh markets in Bangkok, Thailand and the pericarp of GM were dried under shade for 2 days. The pulverized dried plant material (1.0 kg) was extracted with absolute methanol (1 l, two times) for a week at room temperature as described by Chairungrilerd et al. (1996). The extracts were filtered and concentrated to remove the solvent at 75 °C for 4 h and 200 g of CME was yielded eventually. The CME was kept at 4 °C and dissolved with 10% DMSO in RPMI 1640 medium containing 10% FCS for further experiment. A voucher specimen was deposited in forest herbarium of the Royal Forest Department, Bangkok, Thailand.

2.3. Cell culture

SKBR3 cell line was obtained from the American Type Culture Collection (Rockville, MD) and was cultured in RPMI 1640 medium supplemented with 10% (v/v) FCS, 100 mg/l of streptomycin and 100,000 U/l of penicillin G at 37 °C in 5% CO₂ incubator.

2.4. Cell proliferation assay

Serial dilutions of CME (50 μ l) were added into each of 96-well plates, then, cells were plated at a density of

1×10^4 cells/well and incubated for 48 h. After incubation, the medium was removed and cells in each well were incubated with HBSS contained 1 mg/ml MTT for 2 h at 37 °C in 5% CO₂ incubator. MTT solution was then discarded and 50 μ l of isopropanol was added into each well to dissolve insoluble formazan crystal. Plates were then kept agitation for 5 min at room temperature for complete solubilization. The level of colored formazan derivative was analysed on a microplate reader (Molecular Devices, CA) at a wavelength of 590 nm (Moongkarndi et al., 1991; Studzinski, 1995). The percentage of cell viability was calculated according to the following equation.

$$\text{The \% of cell viability} = \frac{\text{OD of treated cells}}{\text{OD of control cells}} \times 100$$

2.5. Determination of morphological changes of cells

2.5.1. Observation of cells by phase contrast microscope

Cells (2×10^5 cells/well) were incubated for 48 h in the absence or presence of CME in 24-well plates. After incubation, the medium was removed and cells in wells were washed once with HBSS. They were observed by phase contrast inverted microscope (Zeiss, Germany) at 400 \times magnification (Chih et al., 2001).

2.5.2. Benzimidazole Ho33342 staining

Cells (2×10^5 cells/well) were incubated for 48 h with CME in 24-well plates. After incubation, Ho33342 (1 μ g/ml) was added to each well and further incubated at 37 °C for 30 min in the dark. Living and apoptotic cells were visualized through blue filter of fluorescence inverted microscope (Zeiss, Germany) at 400 \times magnification (Ramonede and Tomas, 2002).

2.5.3. Propidium iodide (PI) staining

PI can stain the nuclear changes of living and apoptotic cells in the same manner as Ho33342 does. The PI staining was performed as described by Sarker et al. (2000). Briefly, cells (2×10^5 cells/well) were incubated for 48 h with CME in 24-well plates. After incubation, cells were permeabilized with a mixture of acetone:methanol (1:1) at –20 °C for 10 min after treating with extract. Cells were washed with HBSS, then, 200 μ l of 5 μ g/ml PI was added into each well and incubated at 37 °C for 30 min in the dark. Cells were detected by green filter of fluorescence inverted microscope (Zeiss, Germany) at 400 \times magnification (Sarker et al., 2000).

2.6. Detection of DNA fragmentation

DNA fragmentation was analysed by agarose gel electrophoresis as described by Yang et al. (2000) with slight modifications. Cells (3×10^6 cells) were exposed to the extract for 48 h and were gently scraped and harvested by centrifugation. The cell pellets were incubated for 60 min

at 50 °C in 100 μ l lysis buffer (100 mM Tris–HCl pH 8, 100 mM NaCl and 10 mM EDTA). Proteinase K (10 μ l of 20 mg/ml) was added and further incubated for 30 min at 50 °C. RNase (3 μ l of 10 mg/ml) was then added and incubated for 2 h at 50 °C. The DNA was extracted with phenol–chloroform–isoamyl alcohol, subjected to 2.0% of agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light transilluminator (Fotodyne, WI, USA).

2.7. Measurement of ROS production

Intracellular reactive oxygen species (ROS) production was measured in both CME-treated and control cells using DCFH-DA (Chang et al., 2001). Briefly, 2×10^5 cells/well were exposed to CME with various concentrations and different incubation times. After incubation, cells were detached with trypsin–EDTA and washed once with PBS. Treated and control cells were resuspended in 0.5 ml PBS containing 10 μ M DCFH-DA at 37 °C for 30 min and then incubated with 4 mM H₂O₂ (as inducer for ROS production) at 37 °C for 30 min. ROS production of cells were subjected to evaluate by luminescence spectrophotometer (Perkin-Elmer, MA).

2.8. Statistical analysis

The experiments were repeated three to four times and the results were expressed as mean \pm S.D. Statistical analysis was done using two-tailed Student's *t* test and *P* values at a level of 95% confidence limit.

3. Results

3.1. Effect of CME on the proliferation of SKBR3 human breast cell line

The relationship between concentration of CME and their cytotoxic effect on SKBR3 cells was investigated by MTT assay. Cells were treated with CME at concentrations ranging from 0 to 50 μ g/ml for 48 h and then the percentage of cell viability was analysed as described in Section 2. CME from pericarp of GM significantly inhibited the proliferation of SKBR3 cells in a dose-dependent manner (Fig. 1). Similar result was observed when quercetin and paclitaxel were served as a positive control (Moongkarndi et al., 1991; Blajeski et al., 2001). CME at 6.25–50 μ g/ml decreased the proliferation of SKBR3 cells by 20–100% and with an ED₅₀ of 9.25 ± 0.64 μ g/ml.

3.2. Effect of CME on the morphological changes of SKBR3 human breast cancer cell line

After incubation with 20 μ g/ml of CME, morphological alterations in SKBR3 cells were illustrated (Fig. 2B) comparing with control cells (Fig. 2A). Untreated or control cells were cuboid and polygonal in normal shape. Exposure of SKBR3 cells to CME for 48 h led to retraction, rounding and some sensitive cells were detached from the surface. Membrane blebbing (Fig. 2B(a), arrow No. 2) and apoptotic body (Fig. 2B(a), arrow No. 3) were observed by phase contrast inverted microscope. In addition, nuclear fragmentation (Fig. 2B(b and c), arrow No. 5) and nuclear shrinking

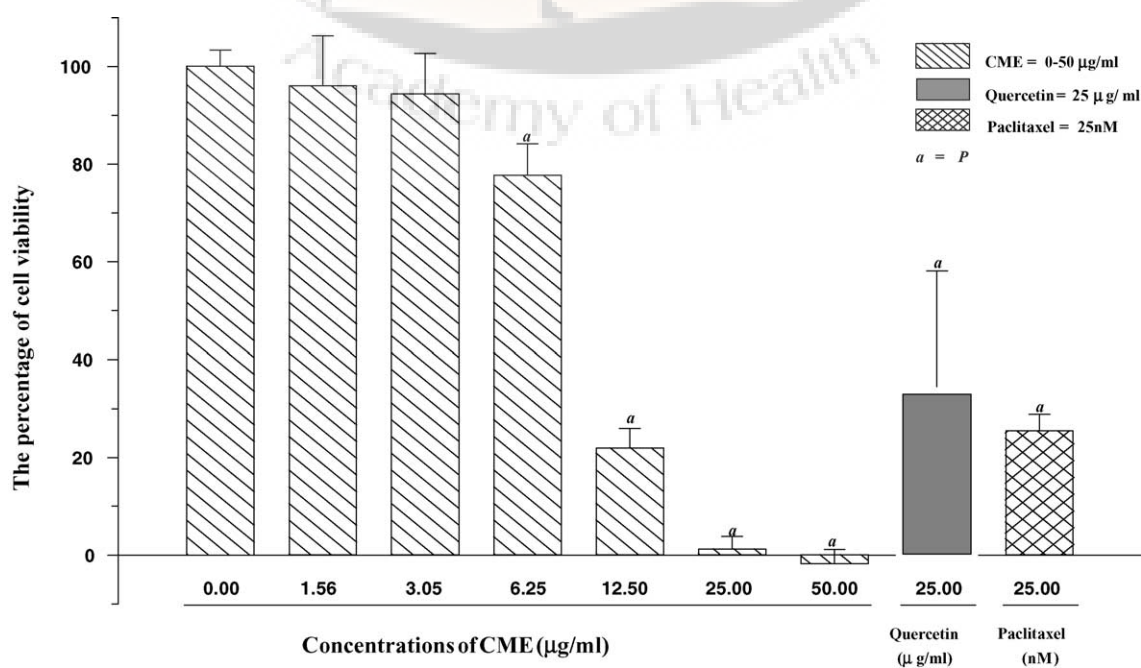


Fig. 1. Effect of CME from GM on the proliferation of SKBR3 cells. The percentage of cell viability was measured by MTT assay. Data represent the means \pm S.D. ($n = 4$).

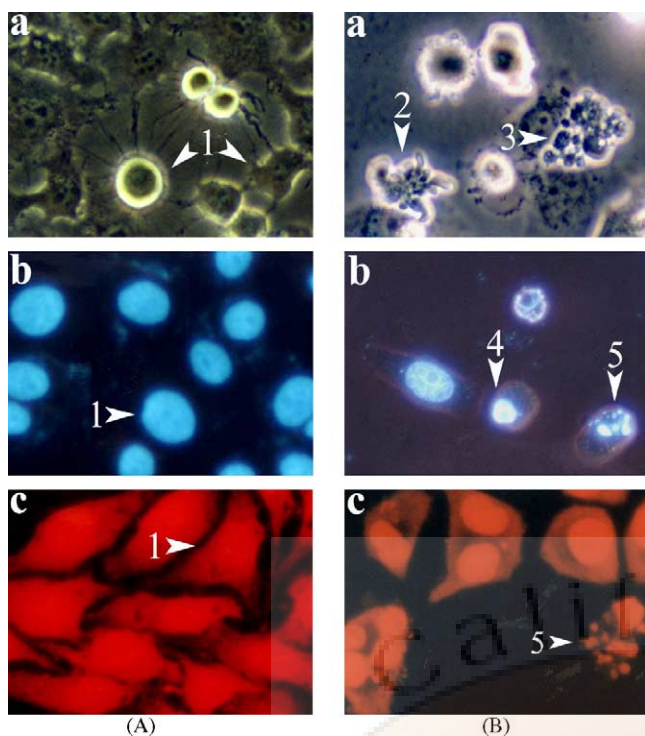


Fig. 2. Morphological alterations of SKBR3 cells following expose to 20 $\mu\text{g/ml}$ of CME for 48 h. (A(a)) Control SKBR3 cells were observed by phase contrast inverted microscope. (A(b)) Control SKBR3 cells were stained by Ho33342. (A(c)) Control SKBR3 cells were stained by PI. (B(a)) CME-treated SKBR3 cells were observed by phase contrast inverted microscope. (B(b)) CME-treated SKBR3 cells were stained by Ho33342. (B(c)) CME-treated SKBR3 cells were stained by PI. 1 \blacktriangleright : normal cells; 2 \blacktriangleright : membrane blebbing; 3 \blacktriangleright : apoptotic body; 4 \blacktriangleright : nuclear shrinking; 5 \blacktriangleright : nuclear fragmentation.

(Fig. 2B(b), arrow No. 4) of SKBR3 cells were illustrated by Ho33342 and PI staining.

3.3. Appearance of DNA ladders in CME-treated cells

The DNA fragmentation of SKBR3 cells (3×10^6 cells) were detected on a 2.0% agarose gel electrophoresis after exposing with 0, 20, 80 and 100 $\mu\text{g/ml}$ of CME for 48 h. At exposure to 100 $\mu\text{g/ml}$ of CME, fragmented DNA was clearly observed in SKBR3 cells (Fig. 3) whereas control cells did not provide ladders. Thereby, it is possible that CME from GM causes apoptosis of SKBR3 cells.

3.4. Effect of CME on the ROS production of SKBR3 human breast cancer cell line

To investigate possible correlation between time and concentration of CME on ROS production, SKBR3 cells were incubated with CME at concentrations ranging from 0 to 40 $\mu\text{g/ml}$ for 24, 48 and 72 h using Vitamin E as a positive control. Intracellular ROS was measured in terms of fluorescence by DCFH-DA. CME from GM could significantly suppressed the intracellular ROS production of SKBR3 cells

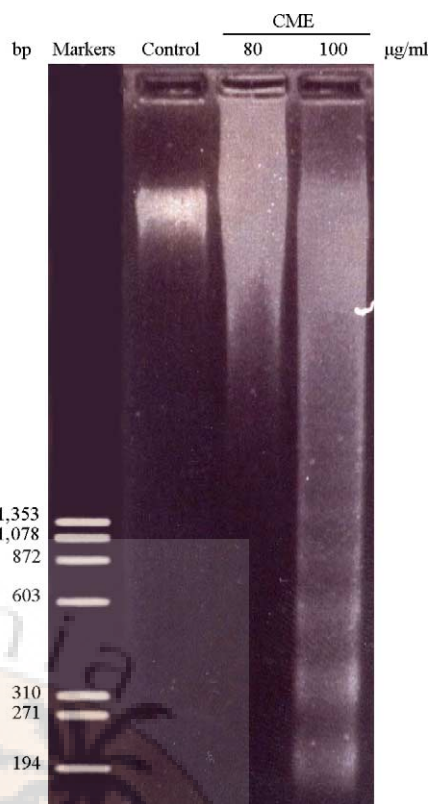


Fig. 3. Effect of CME on DNA fragmentation of SKBR3 cells and ladders were detected by 2.0% agarose gel electrophoresis.

in a dose-dependent manner (Fig. 4). Notably, at 40 $\mu\text{g/ml}$ of CME and incubation time for 48 h, treated cells showed a remarkably increase of ROS level. This case presumably revealed that most cells were induced early apoptosis which caused by oxidative stress. Such condition led to oxidative injury of cells that eventually resulted in cellular component damage and late apoptosis.

4. Discussion and conclusion

Although GM has long been served as traditional medicines, very few authentic scientific studies in field of cancer therapy are available. Recent in vitro studies have shown that many constituents from GM have a wide range of biological actions including antibacterial, antifungal, antihelmith, insecticidal activities (Farnsworth and Bunyapraphatsara, 1992) and anti HIV-1 protease (Chen et al., 1996). Some studies have revealed that pericarp of GM is source of xanthone, mangostin and tannin, etc. Particularly, tannin was found to be an inducer for apoptosis on human leukemia cells (Yang et al., 2000). Moreover, mangostin also inhibited low-density lipoprotein oxidation (Williams et al., 1995).

In this study, we investigated the antiproliferation, antioxidant and induction of apoptosis by CME from pericarp of GM on human breast cancer cell line. We found that CME

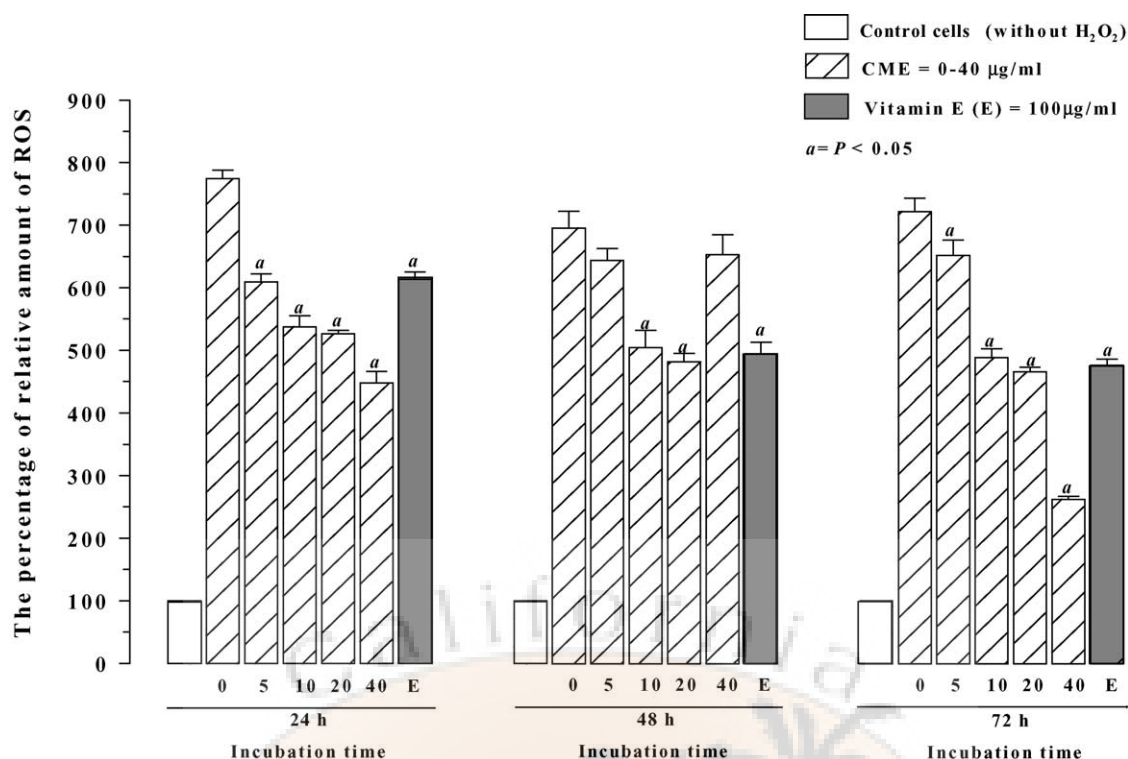


Fig. 4. Effect of CME from GM on ROS production of SKBR3 cells by using DCFH-DA as fluorescence probe. Data represent the means \pm S.D. ($n = 3$).

significantly inhibited the proliferation of breast cancer cells after an incubation period of 48 h and the antiproliferative effect was evaluated by MTT reduction assays. The results presented here showed a concentration-dependent decrease in the percentage of cell viability and at a concentration of 6.25–25 µg/ml of CME was sufficient to effectively inhibit the cell proliferation. Thus, CME displayed the strong antiproliferative activity on breast cancer cells with an ED₅₀ of 9.25 ± 0.64 µg/ml.

To investigate whether apoptosis is involved in the cell death caused by CME on SKBR3 breast cancer cells, we assessed morphological changes and DNA ladder patterns on agarose gel electrophoresis. Morphological analysis of cells with Ho33342 and PI staining strikingly displayed nuclear shrinking, DNA condensation and fragmentation (Fig. 2B(b and c)) after treating cells with 20 µg/ml of CME for 48 h. Moreover, morphological changes were also observed by phase contrast microscope which exhibited cytoplasmic membrane shrinkage, loss of contact with neighboring cells, membrane blebbing and apoptotic body (Fig 2B(a)). In addition, oligonucleosomal DNA fragments (ladders) from cells were exhibited by 2.0% agarose gel electrophoresis after incubation with 100 µg/ml of CME (Fig. 3). These hallmark features of morphological changes suggested that CME from GM caused apoptosis of SKBR3 breast cancer cells.

In this study, we found that CME significantly decrease intracellular ROS production on SKBR3 cells in dose- and time-dependent manner during 24 and 72 h. Although the ROS level was increased by 40 µg/ml of CME at 48 h in-

cubation time and mostly decreased by the same concentration at 72 h incubation time. It was possible that CME at a concentration of 40 µg/ml and with 48 h incubation time, early apoptosis could have been induced in cells. This phenomenon is possible, since the accumulation of intracellular ROS is one of the important processes leading to early apoptosis. Such condition of oxidative stress causes the damage of various cellular component (protein, DNA and other organelles) and finally results in programmed cell death or apoptosis (Wei et al., 2000). Thus, at 40 µg/ml of CME and 72 h incubation time, ROS level was dramatically and decreased since only cell debris remains in well. It appeared that CME at high (40 µg/ml) dose cause apoptosis whereas at low (5 µg/ml) and medium (10–20 µg/ml) doses show antioxidative effects on breast cancer cells. On the other hand, it has been proposed that the excessive production of ROS is not involved in cancer cell proliferation but it is purposed to apoptosis of cells.

In conclusion, the results demonstrated that CME from pericarp of GM have a powerful antiproliferation by inducing apoptotic cell death and a potent antioxidation by inhibiting the intracellular ROS production significantly. Moreover, we assume that determination of ROS level not only measure antioxidation of extract on cells but also measure its induction of apoptosis on cells. These probable properties of GM provide scope of further detail evaluation. Some constituents from GM may serve as a novel powerful antitumour agent and free radical scavenger after further detailed investigation. Moreover, other biological activities and on different

cell lines which are correlated to traditional treatments of GM should be investigated as well such as gastrointestinal tract disorder and chronic infections.

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