Asian Journal of Research in Biochemistry

6(4): 21-30, 2020; Article no.AJRB.57931
ISSN: 2582-0516

Anti-cancer Activity of G*arcinia mangostana* L. and Its Derivatives in Cervical Cancer

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Authors' contributions

This work was carried out in collaboration among all authors. Author SVK performed the laboratory analyses and statistics. Author NRK managed the literature searches. Author SC designed the study, wrote the protocol and draft of the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJRB/2020/v6i430125
Editor(s):
(1) Dr. Asmaa Fathi Moustafa Hamouda, Jazan University, Saudi Arabia.
Reviewers:
(1) D. Malathy, Vels Institute of Science, Technology & Advanced Studies (VISTAS), India.
(2) Inès Karmous, University of Gabes, Tunisia.
(3) Marco Túlio Alves da Silva, São Paulo College, Brazil.
Complete Peer review History: http://www.sdiarticle4.com/review-history/57931

Original Research Article

Received 20 April 2020
Accepted 25 June 2020
Published 03 July 2020

ABSTRACT

The *Garcinia mangostana* Linn, a medicinal plant commonly used in Southeast Asia. The crude extract and isolated metabolites were used to evaluate its potential anti-cancer activity which was compared to methotrexate (MTX) in cervical cancer cell line. The present study involved in using crude mangosteen (CM), γ-mangostin (γ-M), isopropyl mangostin (IPM) and Di-*O*-methyl mangostin (DMM) against the standard anti-neoplastic drug MTX. Cell viability and cytotoxicity by lactate dehydrogenase (LDH) were assessed. Analysis of DNA fragmentation and clonogenic assay further supports the anti-cancer activity of the drugs in *HeLa* cells. The IC_{50} value for CM, γ-M, DMM, IPM and MTX were 13.4µg, 34.84µM, 15.57µM 5.3µM and 16.05µM respectively observed in the present study. This study suggests that the *G. mangostana* and its derivatives have potential anti-cancer activity an *in vitro* study in cervical cancer.

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Keywords: Cervical cancer; Garcinia mangostana; DNA fragmentation; MTT; clonogenic assay; cytotoxicity.

1. INTRODUCTION

Squamous cell carcinoma (SCC) is a second most common cancer of skin, lips, mouth, prostate, urinary bladder, cervix, esophagus and vagina. The ratio of SCC in male to female is 2:1, also estimated that there are approximately 7.6 million or 13% of the world population of mortality due to cancer and the cancer risk may exceed about 26 million populations and 17 million will die of from cancer in 2030 if it is not controlled [1]. Cervical cancer is the second most common cancer among Indian women. In India new cases of cervical cancer detected are 96,922 and deaths 60,078 every year. Chemotherapeutic drugs are often used in combination with an either radiotherapy or surgery or both. The treatment side effect includes nausea, vomiting, diarrhea, hair loss, leucopenia etc. [2]. The incidence increases with age and other socio-demographic factors includes virus, diet, alcohol, tobacco, occupational hazard, sunlight etc. are also contributed to the development of SCC of cervix. Human cancer cell lines are fundamental models, either *in vitro* as monolayer culture or *in vivo* as xenograft in mice, to study the efficacy of therapeutic agents in cancer therapy. *HeLa* was the first cultured cancer cell line, which was derived from cervical cancer cells taken from Henrietta Lacks in 1951 [3]. Since then, several cervical cancer cell lines including SiHa, CaSki, C-33A and ME-180 were established [4]. It is worth to mention that these cell lines do not have equal value as tumor models, the *in vitro* results of drug efficacy experiments are different. For decades, human immortal cancer cell lines have constituted an accessible, easily usable set of biological models with which to investigate cancer treatment and to explore the potential efficacy of anti-cancer drug research. *HeLa* cell line is a suitable cell line model to study cervical cancer *in vitro*. Human papilloma virus (HPVs) is frequently integrated into the cellular DNA in cervical cancers [5] and the two strains of HPV together are responsible for approximately 70% of all cervical cancers [6].

Studies on natural compounds from fruits and vegetables for their health-promoting effects in recent years have received a great attention due to their diverse source of Phytochemicals and daily consumption of fruits and vegetables has been shown to lower the risk of some chronic non-communicable diseases like cardiovascular illness and cancers [7]. Dietary phytochemicals play a vital role in cancer prevention and it is estimated that more than 5000 phytochemicals have been identified to have an anti-proliferative activity [8,9]. In this context, evidence-based molecules for cancer prevention are very well mandatory for practical use. Among them, polyphenols are of great attention as chemopreventive agents because of their anti-oxidative and possible anti-cancer activities [10, 11]. Mangosteen is a tropical fruit from *Garcinia mangostana* L., family of Guttiferae that grow in hot and humid climates. The alternative names are mangostan, manggis, mangis, mang etc. Mangosteen has a source of xanthones, mangostin, tannin, chrysanthemin, gartanin, garcinone, vitamin B1, B2 and vitamin C. Xanthones are rich in mangosteen and its derivatives such as α, β and γ-mangostin have superior biological activity [12]. All parts of the mangosteen can be used in the treatment of various ailments and phytochemical compounds such as oxygenate and a prenylated xanthones are rich in mangosteen than other plants was reported [13]. It was stated to have anti-inflammatory [14], anti-microbial [15], anti-cancer [16], anti-angiogenic [17] and *in vitro* anti-oxidant [18] activities. Xanthones from mangosteen showed multi-targeting pharmacological properties were studied [19]. Cancer represents a public health problem worldwide and the development of new drug molecules are still an urgent need. We previously investigated an anti-proliferative activity of xanthones from *G. mangostana* in human breast cancer and laryngeal carcinoma cell lines, interestingly, γ-mangostin induced mitochondrial dysfunction, cell-cycle arrest and apoptosis in MCF7 and Hep2 cell lines [20]. In this paper, we discussed the mechanism of anti-cancer effect of CM and its derivatives from *G. mangostana* and its possible chemo preventive activity in cervical cancer.

2. MATERIALS AND METHODS

2.1 Cell Culture

The human cervical cancer cell line, *HeLa* was obtained from National Centre for Cell Science (NCCS), Pune, India. Cells were routinely maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum

(Gibco) and incubated at 37°C under 5% CO_2 at high humidity. Inhibitory concentration (IC_{50}) was done by the method of Kuo et al. [21]. Briefly, 48-hour monolayer culture of *HeLa* cells at a concentration of $1x10^4$ cells/well was seeded in 96-well microtiter plate. Different concentrations of CM, γ-M, IPM, DMM and standard anti-neoplastic drug, MTX were mixed in dimethyl sulfoxide (DMSO) and added to the cells. These were incubated at 37°C under 5% CO_2 to derive IC_{50} concentration of the drugs.

2.2 Experimental Design

Study design comprised of six groups; group1: *HeLa* cells; group 2: *HeLa* cells treated with CM; group 3: *HeLa* cells treated with γ-M; group 4: *HeLa* cells treated with IPM; group 5: *HeLa* cells treated with DMM and group 6: *HeLa* cells treated with MTX.

2.3 Trypan Blue Exclusion Assay

Trypan blue exclusion assay was performed by the standard method of Altman et al. [22]. Briefly, 48-hour monolayer culture of cancer cells at a concentration of $1x10^4$ cells/well was seeded in 96-well microtitre plate. Added suitable IC_{50} concentrations of drugs and cells were supplied with 2% DMEM, and incubated at 37°C under 5% CO_2 for 24 hours.

2.4 Cell Viability Assay

Cell viability was estimated using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma- Aldrich, Louis, MO, USA) according to the standard method [23]. This method is based on the reduction of a tetrazolium salt by mitochondrial dehydrogenase enzyme in viable cells. Culture medium was removed after a predetermined time; cells were incubated with 0.2 mL of MTT (5mg/mL) for 3 hours at 37°C. After incubation, the medium was aspirated and the formazan reaction product was dissolved in suitable solvent and measured at 545 nm. The results are expressed as percentage viability.

2.5 Evaluation of Cytotoxicity

Cytotoxicity was assayed through LDH release. In order to evaluate the cytotoxic effects of CM and its derivatives, cells at a density of 2 $x10^4/cm^2$ were plated in 24-well cell culture plate in DMEM medium containing 10% FBS and incubated at 37°C. After suitable time of incubation, supernatants and cell suspension were used for LDH release by standard procedure [24].

2.6 DNA Fragmentation and Clonogenic Assay

The DNA Fragmentation assay was performed by the standard method using agarose gel electrophoresis [25,26]. The clonogenic assay was done based on the standard protocol [27]. This assay is based on the ability of a single cell to grow into a colony. Briefly, 1% agar with 20% FBS was used as a base and 0.7% agarose with 10% FBS along with 2000 cells/plate/0.1mL to top layer. Plates were incubated for 14 days in a CO_2 incubator; colonies were fixed with glutaraldehyde (6.0% v/v), stained with crystal violet (0.5% w/v) and counted using a stereomicroscope.

$$Calculation = \frac{Number\ of\ clones\ in\ treated\ wells}{Number\ of\ clones\ in\ control\ wells} \times 100$$

2.7 Statistical Analysis

Statistical analysis was done using ANOVA. $p<0.05$ was considered as statistically significant.

3. RESULTS

IC_{50} for crude mangosteen and its derivatives was shown in Fig. 1. The IC_{50} for CM, γ-M IPM, DMM and MTX were 13.4µg, 5.30µM, 34.84µM, 15.57µM and 16.05µM were obtained for the present study.

Fig. 2 shows the IC_{50} analysis for CM and its derivatives with solvent DMSO. There was no significant change in cell number for DMSO treated group in the present study. DMSO is a standard solvent used to dissolve the drugs in cell culture studies. Trypan blue exclusion assay for CM and its derivatives are shown in Fig. 3. The cell number was decreased significantly ($p<0.001$) in group 2 to 6 when compared to group 1. Cell viability was evaluated through MTT assay and it is shown in Fig. 4. Considering 100% viability in group 1, the drug treated groups showed significant decrease in group 2, 4 & 5 ($p<0.05$) and group 3 & 6 ($p<0.01$).

The LDH activity in cell suspension and cell supernatant is given in Fig. 5. The cancer cell itself produce cytotoxicity, this was observed in the present study. The LDH level was significantly increased in group 2 to 5 ($p<0.01$)

and group 6 ($p<0.05$) when compared to group 1. The LDH level in cell supernatant was significantly decreased in group 2 to 5 ($p<0.01$) and group 6 ($p<0.05$) when compared to group 1.

The results of DNA fragmentation was shown in Fig. 6. DNA fragmentation was observed in group 2 to 5 whereas group 6 did not show any DNA fragmentation.

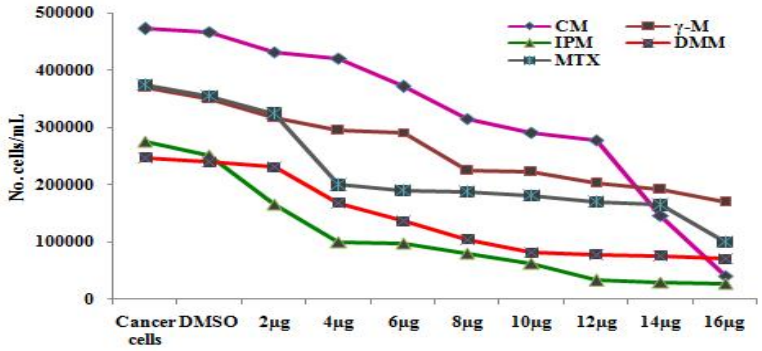

Fig. 1. IC_{50} analysis

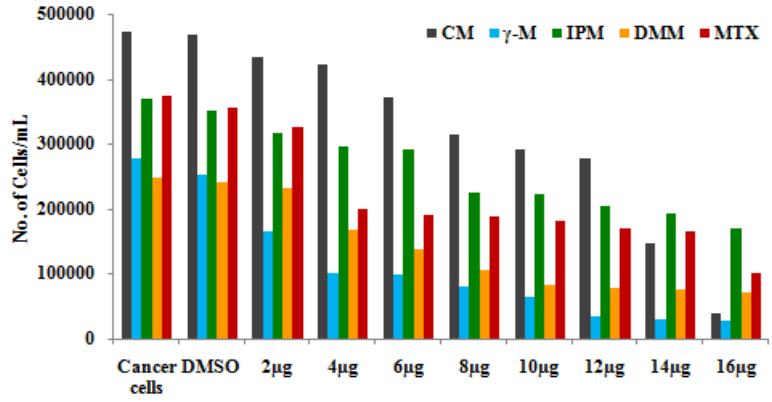

Fig. 2. IC_{50} analysis of CM and its derivatives

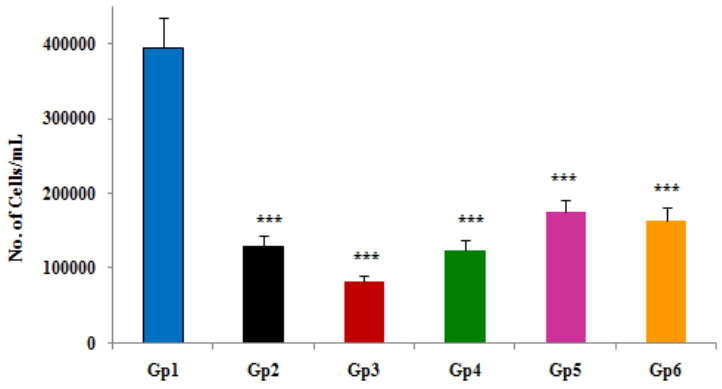

Fig. 3. Trypan blue exclusion assay

*Gp 1- HeLa cells; Gp 2- HeLa cells + CM; Gp 3- HeLa cells + γ-M; Gp 4- HeLa cells + IPM; Gp 5- HeLa cells + DMM; Gp 6- HeLa cells + MTX; ***p<0.001*

Clonogenic assay was depicted in Fig. 7. This is one of the important confirmatory tests used to study the anti-cancer efficacy of the drugs. Morphologies and viabilities of migratory abilities of *HeLa* cells were analyzed using this assay. Studied the percentage control of cell growth as a colonies formed from day 7 to day 14. The present study showed that the growth control of 79% for CM, 97.6% for γ-M, 88.0% for IPM, 89.4% for DMM and 91.3% for MTX for 14th day when compared to 7th day.

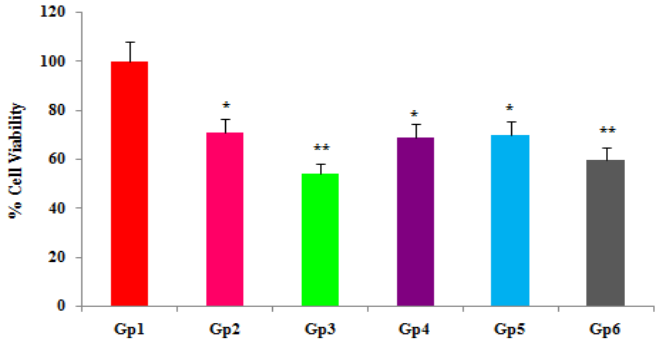

Fig. 4. Cell viability assay

*Gp 1- HeLa cells; Gp 2- HeLa cells + CM; Gp 3- HeLa cells + γ-M; Gp 4- HeLa cells + IPM; Gp 5- HeLa cells + DMM; Gp 6- HeLa cells + MTX; *p<0.05; **p<0.01*

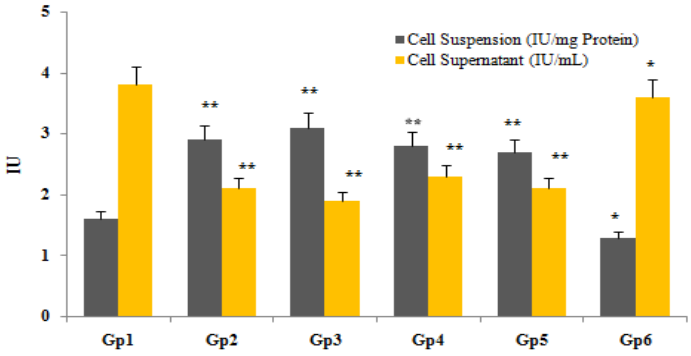

Fig. 5. Cell cytotoxicity assay

*Gp 1- HeLa cells; Gp 2- HeLa cells + CM; Gp 3- HeLa cells + γ-M; Gp 4- HeLa cells + IPM; Gp 5- HeLa cells + DMM; Gp 6- HeLa cells + MTX; *p<0.05; **p<0.01*

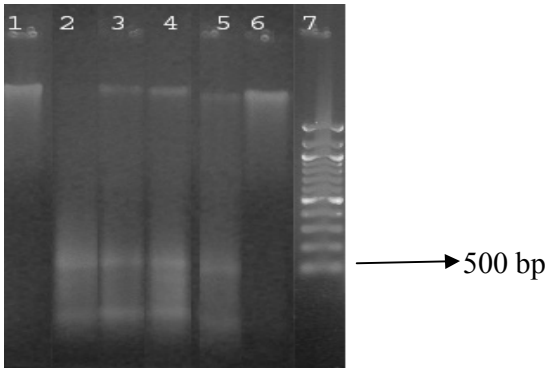

Fig. 6. DNA fragmentation assay

Lane 1- HeLa cells; Lane 2- HeLa cells + CM; Lane 3- HeLa cells + γ-M; Lane 4- HeLa cells + IPM; Lane 5- HeLa cells + DMM; Lane 6- HeLa cells + MTX; Lane 7- Marker

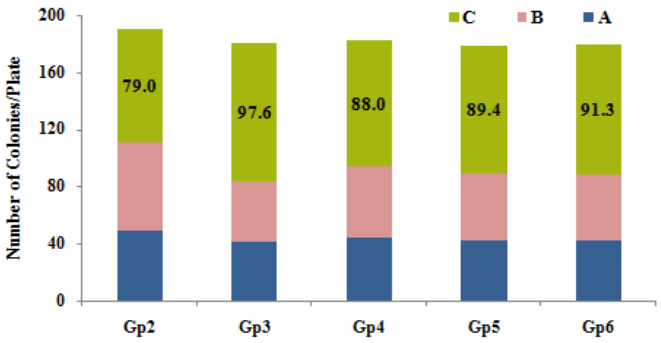

Fig. 7. Clonogenic assay

Gp 1- HeLa cells; Gp 2- HeLa cells + CM; Gp 3- HeLa cells + γ-M; Gp 4- HeLa cells + IPM; Gp 5- HeLa cells + DMM; Gp 6- HeLa cells + MTX; A-7thday; B-14thday; C-% Growth control between 7th & 14th day

4. DISCUSSION

The prenylated xanthones from the pericarps of mangosteen were examined for the effect on the growth of human cervical cancer cells. Trypan blue stains dead cells and live cells appear translucent depending on plasma membrane integrity and permeability. The trypan blue exclusion assay showed that 66.92%, 79.04%, 68.43%, 55.81% and 58.33% for CM, γ-M, IPM, DMM and MTX were respectively when compared to untreated cancer cells. Several lines of evidence from numerous *in vitro* and *in vivo* studies have confirmed that xanthones inhibit proliferation of a wide range of human tumor cell types by modulating various targets and signal transduction pathways. Cancer prevention using non-toxic chemical entities, generally known as "chemoprevention" is a more pragmatic and primary step for the management of malignant diseases. Due to limitations of existing therapies, natural products may serve as chemoprevention regimen and/or novel adjunctive agents to fill a significant need in the effective, safe, and less invasive treatment of cancer. IPM derivative of the present study showed a superior and potent inhibitory activity, this might be due to the presence of more number of hydroxyl groups in its structure. The percentage viability was done using MTT assay considering 100% for group 1, the other groups showed that 71%, 54%, 69%, 70% and 60% for CM, γ-M, IPM, DMM and MTX respectively. The percentage control in cell growth was higher for γ-M followed by MTX, IPM, DMM and CM in the present study. In addition, xanthones especially γ-M could inhibit cell growth via decreasing aromatase activity which is required for the growth of estrogen-dependent breast cancer [28]. As a whole the anti-tumor activity of xanthones from *G. mangostana* includes suppression of tumor cell proliferation, cell-cycle arrest, induction of apoptosis and differentiation, reduction in inflammation, inhibition of adhesion, invasion and metastasis [29-31]. A reduction in cell number in MTX treated groups of present study might be due to inhibition of DNA synthesis, either by blocking the formation of pyrimidine nucleotides via reduction of folate reductase enzyme or inhibiting the formation of tetrahydrofolate.

Cell cytotoxicity was observed through LDH release and it is a stable cytoplasmic enzyme present in all cells and rapidly released into the cell supernatant upon damage of the plasma membrane. Therefore, the release of LDH from cells leads to reduced levels in cell suspension of all drug treated groups whereas in cell supernatant the levels were increased. This change may be due to mangosteen and its derivatives control most of the enzymes involved in the regulatory mechanism of cancer [32-34]. The prenyl group of mangosteen is considered to be implicated in the internalization into the cell, which inturn leads to interaction with the signal transduction molecules and the proteins involved in mitochondrial permeability transition [35,36] which was evident in CM and its derivative treated groups of the present study. Studies revealed that, xanthones not only inhibit the proliferation of tumor cells but also induces cell death through activation of caspase cascade in *in vitro* [37]. The cytotoxicity of MTX results from three important actions: inhibition of dihydroxy folate reductase (DHFR), inhibition of thymidylate synthase, and alteration of the transport of reduced folates. The affinity of DHFR to MTX is

far greater than its affinity for folic acid or dihydrofolic acid.

Apoptosis is an innate mechanism of eukaryotic cell suicide, which plays a major role in many physiological and pathological processes. During apoptosis, a series of reorganization occurs in the cell: chromatin condensation, loss of cell volume and membrane blebbing is some of the most evident morphological changes of apoptotic cells. Responsible for DNA cleavage is believed to be an endogenous Ca^{2+} and Mg^{2+}-dependent endonuclease able to break double stranded DNA at inter nucleosomal sites [38]. Therefore, apoptotic DNA cleavage results in characteristic fragments of oligonucleosomal size (>500 bp). The typical ladder pattern of DNA fragmentation was observed in the present study for CM and its derivatives. No DNA fragmentation was noticed in MTX treated group which indicates that MTX is an anti-metabolite which controls cell proliferation through folate reductase pathway not through apoptotic pathway which was evident in the present study. Several studies have showed that clonogenic assay may be carried out 1 to 3 weeks. In our present study, the confluent colonies were found upto two weeks, therefore, the percentage cell growth control in the form of colonies formed between day 7 to day 14 which showed that 97.6<91.3<89.4<88.0<79.0 for γ-M<MTX<IPM<DMM<CM respectively. MTX competitively inhibits dihydrofolate reductase, an enzyme that participates in the tetrahydrofolate synthesis. MTX is still used in the treatment of a variety of tumors, including acute lymphocytic leukemia [39], breast cancer [40], osteosarcoma [41], primary central nervous system lymphoma [42] and head and neck cancer [43]. This metabolic step is essential for de novo synthesis of thymidine nucleotides for DNA synthesis. DHFR was the first enzyme to be identified as a cellular target for the antifolates aminopterin and MTX [44]. The latter exhibits its anti-cancer effect by almost irreversible inhibition of DHFR, with subsequent disruption of purine and pyrimidine synthesis [45]. MTX can induce apoptosis through both mitochondrial pathway and p53 dependent pathway. Another major pathway for the induction of apoptosis is the mitochondrial pathway or the intrinsic pathway [46]. The ant-cancer mechanism of MTX acts through initiation of p53-dependent apoptosis. MTX can induce p53 acetylation at Lys373/Lys382 and phosphorylation at Ser15/Ser392 [47]. From the above literature, the MTX acts as both p53 and DHFR pathway. Therefore MTX was selected for this study to evaluate its anti-cancer activity. Hence the present study showed that γ-M has superior anti-cancer activity followed by IPM than CM. Therefore further studies are required to evaluate anti-cancer activity of xanthones from mangosteen and MTX with caspase mediated pathway such as both intrinsic and extrinsic in cervical cancer cell lines might help to understand the exact mechanism behind these drugs.

5. CONCLUSION

G. mangostana have become very popular fruit and dietary supplement and they are the rich source of various remarkable natural compounds of xanthones with multi-targeting biological properties like anti-oxidant, anti-cancer, anti-ulcer anti-microbial and anti-inflammatory activities. Some xanthones were synthesized and modified by several techniques to create novel compounds that may improve the efficacy. However, the relation between mangosteen and its derivatives has not been extensively studied in cancer. Further research is needed to study on the exact mechanisms of action and this can be extrapolated to new drug discoveries. Increasing awareness on the utilization of this fruit for public benefits is highly encouraged. Proteomic and genomic studies are needed to determine the efficacy of this fruit extract to serve as natural anti-cancer agent in treatment of cancer.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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