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Functional Structure/Activity Relationships

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Differential effects of quercetin and its two derivatives (isorhamnetin and isorhamnetin-3- glucuronide) in inhibiting proliferation of human breast cancer MCF-7 cells

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ABSTRCT: Quercetin (Que) has consistently been reported to be useful cytotoxic compound *in vivo and in vitro,* but little is known on its metabolites. Here we examined and compared cytotoxic effect of Que and its water-soluble metabolites, isorhamnetin (IS) and isorhamnetin-3-glucuronide (I3G) in human breast cancer MCF-7 cells to uncover their tumor-inhibitory mechanism and structure-function relationship. The results showed that Que, IS and I3G could dose-dependently inhibit the growth of MCF-7 cells, and the cytotoxic effect was ranked as Que > IS > I3G. Furthermore, Que, IS and I3G mediated the cell-cycle arrest principally in S phase, followed by the decrease in the number of G0/G1 and G2/M, and 70.8%, 68.9% and 49.8% MCF-7 tumor cells entered early phase apotosis when treated with 100 μM Que, IS and I3G for 48 h, respectively. Moreover, induction of apoptosis by Que, IS and I3G were accompanied with the marginal generation of intracellular ROS. Given these results, Que, IS and I3G possess strong cytotoxic effect through a ROS-dependent apoptosis pathway in MCF-7 cells.

KEYWORDS: *Quercetin, isorhamnetin, cytotoxicity, cell circle, apoptosis*

INTRODUCTION

Breast cancer is the leading cause of tumor death among women,[1,2] and more and more reports consistently show that regular consumption of fruits and vegetables is strongly associated with reduced risk of tumor.[3,4] Furthermore, many benefits of fruits and vegetables are shown to be due to the ingestion of vast flavonoids, a type of functional compounds with a common phenylbenzopyrone structure (C6-C3-C6).[5,6] As one of the primary flavonoids, quercetin (3,3′,4′,5,7-pentahydroxyflavone) has been reported to have anti-tumor effect on many tumor cells, which may be related to catechol moiety in B ring and free hydroxyl groups in the quercetin structure.[7-11]

Recent studies have showed that quercetin can be metabolized into various sulphated, glucuronidated and methylated forms in different organs, such as liver, kidney, colon and small intestine, and its metabolites may still act as antioxidants with higher hydrophily.[12] Our previous studies have also showed that QS (quercetin-5',8-disulfonate) can possess remarkably high anti-tumor activity in human breast cancer MCF-7 cells,[11] indicating that sulfated metabolites of quercetin may play an important role in cytotoxic effects. For this reason, whether the methylated-, methylated- and glucuronidated- metabolites of quercetin also play a crucial role in quercetin-induced biological effects remains poorly understood, and few studies reported the cytotoxic effect of them. Accordingly, it is necessary to further study the anti-tumor effect and their molecular mechanism of methylated quercetin and other metabolites. Significantly, it is interesting to note that the difference in cytotoxic activities between quercetin and its metabolites may also help understand the structure-activity relationship of the tested compounds.[13]

With this in mind, in present study we chose the water-solubility metabolites Isorhamnetin (IS) and Isorhamnetin-3-glucuronide (I3G) (Fig. 1), which were synthesized by Paul W. Needs,[14] to evaluate the cytotoxic effect and make clear the structure-activity relationship of them by investigating and comparing the cytotoxicity, cell circle distribution, apoptosis, cellular morphology and intracellular ROS generation in human breast cancer MCF-7 cell line, and ultimately purify the molecular mechanism. These findings can help understand the structure-activity relationship in tumor-inhibitory effects and have important implications for the potential use of the quercetin and its metabolites in the treatment of human breast cancer.

MATERIALS AND METHODS

Chemicals and reagents

The quercetin was the product of National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and its purity (>98%) was verified by UPLC. IS and I3G (Fig. 1) were synthesized and presented as solid sodium salts form by Paul W. Needs.[14] EDTA and Triton X-100 were the products of Sinopharm Chemical Reagents Co., Ltd (Shanghai, China). 3-(4,5-Dimethylthi-azol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Rnase-A and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Dihydroethidium (DHE) and dichlorofluorescein diacetate (DCFH-DA) were obtained from BestBio Co. (Shanghai, China). Millipore Milli Q-plus System (Millipore, Bedford, MA, USA) was used to prepare deionized water. The other reagents were all analytical reagents.

Cell lines and culture

Human breast carcinoma MCF-7 cells were products of Cell Bank of Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were grown in RPMI-1640 medium in a humidified incubator at 37ºC with 5% CO_2, and the medium consists of 100 U/mL penicillin, 10% heat-inactivated fetal bovine serum (FBS) and 100 μg/mL streptomycin.[11] To improve the reliability of the data, we repeated all experiments for three times at least per experimental point.

MTT assay

Determination of live cell numbers is often used to assess the rate of cell proliferation caused by drugs and cytotoxic agents. Among all non-radioactive viability assays, MTT assay developed by Mossman is one of the most versatile and popular assays. MTT is a tetrazolium salt that is turned into a purple formazan product after reduction by mitochondrial enzymes that are only present in metabolically active live cells, not in dead cells.[11] The cells were seeded and grew in 96-well plates at concentration of 3×10^5 cells/well in 100 μL medium for 24 h (the cells were grew to 70% confluence). Then we used medium consists of different concentrations (0, 25, 50 and 100 μM) of Que, IS, I3G or 5-fluorouracil (5-Fu, 100 μM) to treat the cells, respectively. The 5-Fu is used as a positive control in cell experiments. In this study, we added 10 μL of MTT (5 mg/mL) in PBS solution to each well. After blending them, we further incubated the plate. 100 μL of solution containing 0.01 M HCI, 5% isobutyl alcohol and 10% SDS (pH 4.8) was added to each well in 4 h, mixed and put in incubator for one night. The absorbancy was observed at 570 nm using a microplate spectrophotometer (RT6000, Guangdong, China). The viability of cells was calculated with the following formula: cell viability (%) = $OD_{test}/OD_{control} \times 100\%$, and the compounds IC_{50} (50% inhibition

concentration) values were counted using the Origin 7.0 software.

LDH assay for cytotoxicity

Lactate dehydrogenase (LDH) is a cytosolic enzyme present in many different types of cells. When the plasma membrane is damaged, LDH is released into cell culture media. The released LDH can be quantified by a coupled enzymatic reaction.[11] The cytotoxic effects of Que, IS and I3G on human breast MCF-7 cells were investigated by LDH assay. In this study, LDH kit (Jiancheng BioEngineering, Nanjing, China) was used to test cellular membrane damage of MCF-7 cells in response to Que, IS and I3G (0, 25, 50 and 100 μM) treatments as outlined by manufacture with minor modifications. After incubation at room temperature for 30 minutes, reactions are stopped and 20 μL of culture supernatant was took out for the activity analysis of extracellular LDH, which could catalyze the lactate turn into pyruvate. Then the culture supernatant was reacted with 2,4-dinitrophenylhydrazine to make the basic solution present brownish red color, and LDH activity was determined by spectrophotometric absorbance at 450 nm.

Morphological study

Regularly examining the morphology (shape and appearance) of the cells in culture is essential for successful cell culture experiments.[15] In this study, the morphological study of MCF-7 cells treated with Que, IS and I3G was investigated using an Inverted Fluorescence Microscope. Human breast cancer MCF-7 cells were seeded onto a glass slide and treated with Que, IS and I3G for 48 h. After washing at least two times with ice-cold PBS, they were blended with 4% (V/V) formaldehyde in PBS and then washed PBS. After the cells were stained with 1 mg/mL Hoechst 33258 in PBS at 37ºC for 15 min, we examined the morphology

using a fluorescence microscope (Leica DMIL LED, Leica, Germany) with an excitation wavelength of 345 nm through the filter of 420 nm.

Assessment of cell apoptosis

Apoptosis is a distinct form of cell death controlled by an internally encoded suicide program.[16] The extent of apoptosis was investigated by Annexin V-FITC/PI double staining assay. In this study, an Annexin V-FITC/PI Apoptosis Detection Kit (BestBio, Shanghai, China) was used to determine early and late apoptotic changes in MCF-7 cells. MCF-7 cells (3×10^5) were collected, washed with PBS for two times and then suspended in 400 μL of binding buffer (adding 5 μL of annexin V-FITC and 10 μL of PI). After incubating for 10 min at 2-8 ºC in the dark, we used a GUAVA® easy Cyte™ 8HT flow cytometry (Millipore Corporation, Billerica, MA, USA) to analyze the samples. Then we counted the number of annexin V-FITC-positive and PI-positive of cells in each field. In order to ensure the reliability of the data, we independently did the whole experiments for three times at least.

Cell circle analysis by flow cytometry

DNA content assay for cell circle is a classical method that frequently employs flow cytometry to distinguish cells in different phases of the cell circle.[17] For the purpose of studying the relationship between growth inhibitory effect of Que, IS and I3G and cell circle arrest, we treated MCF-7 cells with Que, IS and I3G for 48 h and then examined cell circle phase distribution of PI-stained by using flow cytometry. MCF-7 cells (3×10^5) were seeded in 6-well flat-bottomed plates and grown overnight until they reached 80% concentration, and the medium was changed after 24 h. After treatment with Que, IS and I3G (0, 50, 100 μM) for 48 h, we collected the detached cells in culture, and then combined them with the remaining

adherent cells that were detached by brief trypsinization (0.25% trypsin-EDTA, Sigma-Aldrich). After mixing and washing the cell pellets in 75% ethanol with PBS, we resuspended them in PBS (1 mL) containing 1 mg/mL RNase (Sigma-Aldrich) and 50 μg/mL PI (Sigma-Aldrich). The cells were incubated in the dark for 30 min at 26ºC, and then investigated by the GUAVA® easy Cyte™ 8HT flow cytometry (Millipore Corporation, Billerica, MA, USA).

Measurement of ROS

Generation of intracellular ROS was assessed using dichlorofluorescein diacetate probes.[16] MCF-7 cells were seeded in 12-well plates, and then incubated with Que, IS and I3G for 24 h. After detaching with trypsin-EDTA, we washed the cells with PBS for twice and then incubated them with 5 μM DCFH-DA for 30 min at 37ºC. Then flow cytometry was used to determine the fluoresence intensity of MCF 7 cells.

Statistical analysis

All data are expressed as mean ± SD of three independent experiments. The significant difference from the respective control for each experimental group was examined by one-way analysis of variance (ANOVA) using SPSS 19.0 software. A value of $p < 0.05$ is considered statistically significant and a value of $p<0.01$ means extremely significant difference.

RESULTS

Growth-inhibitory effects of quercetin, IS and I3G on MCF-7 cells

To identify the growth-inhibitory effects of quercetin and its metabolites, we cultured MCF-7 cells treated with the indicated concentrations of Que, IS and I3G at 25, 50, 100 μM for 48 h.

As shown in Fig. 2A, a significant growth-inhibitory effect induced by 25 μM of Que, IS and I3G was observed as compared to the untreated control cells (p<0.01), and a further decrease in the percentage of MCF-7 living cells was observed as the concentrations of Que, IS and I3G increased to 100 μM, indicating that the inhibition was in a dose-dependent manner. After the MCF-7 cells were treated with Que, IS and I3G at the high dose of 100 μM for 48 h, MCF-7 cells viability was markedly decreased to 33.1%, 34.2% and 40.7% in comparison with the control group, respectively (p<0.01). More interestingly, the highest concentration of Que (100 μM) and IS (100 μM) exhibited similar effect with the same concentration of 5-Fu, suggesting that Que, IS and I3G all could exhibit high tumor-inhibitory effect in human breast cancer MCF-7 cells, and this effect was sort as follows: Que > IS > I3G, which might be related to the different structure of metabolites.

Cytotoxicity of quercetin and its metabolites on MCF-7 cells

The release of LDH can be regarded as an index of the integrity of cell membrane necrosis in response to cytotoxic efficiency, and it can be detected by colorimetric assay.[18] Herein, we evaluated LDH release to evaluate the cytotoxicity of Que, IS and I3G (0, 25, 50 and 100 μM) on MCF-7 cells after 48 h of incubation. As can be seen in Fig. 2B, when treated with Que, IS and I3G at the concentration of 25 μM for 48 h, the LDH release of MCF-7 cells was 957 U/L, 942 U/L and 880 U/L, which were 8-10 times higher than that of control group (100 U/L, p<0.01). Along with the increase of quercetin and metabolites concentration, the LDH release of MCF-7 cells represented a significant improvement, indicating that the cytotoxicity effects of Que, IS and I3G in MCF-7 cells were in a dose-dependent manner. When treated with Que, IS and I3G at the high dose of 100 μM for 48 h, LDH release of MCF-7 cells was markedly

increased to 1390 U/L, 1359 U/L and 1279 U/L, which was 12-14 times higher than that of control group (100 U/L, $p<0.01$), respectively and this effect was similar to that of the same concentration of 5-Fu (1599 U/L, $p>0.05$), suggesting the induction of cell membrane injury. Similarly, the cytotoxicity of these compounds on MCF-7 cells could be ranked as Que > IS > I3G, indicating that methylation in 3'-position of Que could decrease the cytotoxicity of Que in MCF-7 cells, and glucuronidation could further decrease the tumor-inhibitory effect of IS (methylation of quercetin in 3'-position). This sensitivity of MCF-7 tumor cells to quercetin and its metabolites led to further examination on the mechanism of antiproliferative effects of them.

Morphological study

After treated with Que, IS and I3G (100 μM), MCF-7 cells were incubated for 48 h and observed the morphological characteristics with an Inverted Fluorescence. As seen in Fig. 2C, untreated control cells grew well, but the tumour cells treated with Que, IS and I3G were gradually reduced, and cells fusion, shrinkage, nuclear condensation, apoptotic body and lysis appeared, which were similar to that of 5-Fu treated MCF-7 cells. The morphology assay results indicated that similar with quercetin, IS and I3G were anti-tumor compounds as well, and their anti-tumor effect can be ranked as Que > IS > I3G, which were consistent with that of the MTT assay and the LDH assay.

Effects of quercetin and its metabolites on cell apoptosis in MCF-7 cells

Apoptosis is a process of programmed cell death that occurs in multicellular organisms, and always be considered as the preferred way to eliminate tumor cells.[16] MCF-7 cells apoptosis was measured by flow cytometry using annexin V-FITC and PI labeling.[18] As displayed in Fig. 3A and B, in untreated control groups, 95.8% of MCF-7 cells were in normal state and almost

no apoptotic nuclei were observed. When treated the MCF-7 cells with 25 μM Que, IS and I3G for 48 h, 36.6%, 35.3% and 16.8% of MCF-7 cells transformed into apoptotic state, which were significant higher than that of control group (1.3%), respectively (p<0.01). Furthermore, with the increase of concentrations, Que, IS and I3G dose-dependently induced 70.8%, 68.9% and 49.8% of MCF-7 cells to transform into apoptotic state. Meanwhile, the cytotoxic effects of IS and I3G were similar to that of 5-Fu (75.2%, p>0.05). Consistent with MTT, LDH and morphology analysis, Que, IS and I3G were proved to induce cell apoptosis in a dose-dependent manner, and the effects could be ranked as Que > IS > I3G, suggesting that methylated and methylated-glucuronidated complex metabolites of quercetin could decrease its pro-apoptosis effect in MCF-7 cells in varying degree.

Quercetin and its metabolites induced cell circle arrest in MCF-7 tumor cells

For the purpose of studying the relationship between growth inhibitory effects of the tested flavonoids and cell circle arrest, MCF-7 cells were treated with Que, IS and I3G for 48 h. The cell circle phase distribution of PI-stained cells was examined by using flow cytometry. As is shown in Fig. 4A and Fig. 4B, after treated with Que, IS and I3G at 50 μM, a significant amount number of MCF-7 cells accumulated at the S-phase, corresponding to DNA synthesis, from 5.7% to 38.1%, 34.5% and 25.2% (p<0.01), accompanied by a decrease in the G0/G1 and G2/M cells, respectively. After 48 h of treatment with 100 μM of Que, IS and I3G, a further increasing arrest in the S-phase of MCF-7 cell circle was observed (p<0.01), which was similar with that of the same concentration of 5-Fu (p>0.05).

ROS was involved in quercetin- or its metabolites-induced apoptosis in MCF-7 cells

Mitochondrial ROS production is a crucial early driver of cell injury, and has been

considered to have a big relationship with the induction of apoptosis.[19,20] To investigate whether intracellular ROS is related to the apoptosis induced by Que, IS and I3G, we determined the ROS level by using flow cytometry. As shown in Fig. 5A and B, after treated with Que, IS and I3G at 25 μM for 12 h, the accumulation of O_2^- in MCF-7 cells was 58.8%, 50.0% and 44.7%, which was significantly higher than that of control cells (6.03%, $p<0.01$), respectively. With an increase in Que, IS and I3G concentrations, a dose-dependent effect was further observed. The accumulation of O_2^- in a high concentration (100 μM) of Que, IS and I3G treated cells was significantly increased to 84.1%, 77.6% and 60.7%, which was similar to that of the same concentration of 5-Fu ($p>0.05$), respectively. Similar results could be observed from the accumulation of H_2O_2, compared with untreated MCF-7 cells, and the accumulation of H_2O_2 in Que, IS and I3G groups at 25 μM was significantly elevated to 48.9%, 36.0% and 20.0% (Fig. 5C and D). When concentrations increased to 100 μM, the accumulation of H_2O_2 was dose-dependently increased to 68.1%, 55.8% and 43.1%, respectively ($p<0.01$). These results indicated that Que, IS and I3G could induce apoptosis through the increasing of intracellular oxidative stress of MCF-7 tumor cells.

Growth-inhibitory effects and cytotoxicity of Que, IS and I3G on normal mammary epithelial cell H184B5F5/M10 cells

Similar with MCF-7 cells, the cytotoxic effects of Que, IS and I3G on normal mammary epithelial cell H184B5F5/M10 cells were also determined using the MTT assay and LDH assay, respectively. Just as shown in Fig.6 (A) and (B), cytotoxic effects of Que, IS and I3G on H184B5F5/M10 normal mammary epithelial cells were not observed in the same test concentrations with MCF-7 cells, indicating that Que, IS and I3G had no growth-inhibitory

effects and cytotoxicity in the normal mammary epithelial H184B5F5/M10 cells.

DISCUSSION

Quercetin (3,3',4',5,7-pentahydroxyflavone) is one of the most widespread flavonoids and was regarded as a promising compound in tumor prevention.[21,22] *In vivo*, absorbed Que is rapidly metabolized to various methylated, glucuronidated and sulfated forms in different organs, such as liver, kidney, colon and small intestine. The metabolites include Que, Isorhamnetin (IS), Isorhamnetin-3-glucoside(I3G), Quercetin aglycone(QA), quercetin 3-glucoside(Q3G), 3'-O-methylated quercetin, 4'-O-methylated quercetin, quercetin-3′-sulfate(Q3'S) and so on.[23, 24] Among these metabolites, IS and I3G (Fig. 1) are very important metabolites for quercetin and they are likely to possess biological properties different from parent quercetin, making it significant to examine their anti-tumor activities and investigate the relationship between structure and function.[23] Nevertheless, there is a paucity of research on the issue with anti-tumor effects of quercetin metabolites. Consequently, in this study we mainly tested and compared the anti-tumor effects of Que, IS and I3G, to figure out the mechanism and try to reveal the structure-activity relationship of them by testing growth-inhibitory effects, cytotoxicity, cell circle effects and ROS level in human breast cancer MCF-7 cells.

IS and I3G both inhibited the growth of MCF-7 cell, and the effect can be ranked as Que > IS > I3G (Fig. 2). Notably, we firstly demonstrated an interesting phenomenon that similar with parent quercetin, structurally related metabolites IS and I3G possessed strong inhibitory effects on human breast cancer MCF-7 cells in a dose-dependent manner, and their inhibitory

activities were similar with positive 5-Fu. Apoptosis is a process of programmed cell death that occurs in multicellular organisms.[25] The results of annexin V/PI co-staining assays in this study clearly showed that Que, IS and I3G could induce MCF-7 cells apoptosis and necrosis, and the activities can be ranked as Que > IS > I3G (Fig. 3). And these results were consist with that of biochemical and morphological assay, which showed that in Que, IS and I3G treatment groups, cell shrinkage, chromatin condensation, inter nucleosomal DNA fragmentation, and formation of "apoptotic bodies" appeared in MCF-7 cells (Fig. 2C). Mounting evidence suggests that apoptosis have a big relationship with cell circle and apoptosis may be induced by cell circle disruption.[26,27] Similar with some previous reports,[28] our cell circle essay indicated that Que, IS and I3G exhibited effective cell growth inhibition by accumulating cells in S-phase, decreasing the MCF-7 cells number of G2/M and G0/G (Fig. 4). To conclusion, this finding suggests that quercetin and its metabolites IS and I3G inhibit hyperplasia of tumor cells mainly by arresting the cells in the S-phase and decreasing the number of G0/G1 and G2/M cells in the cell circle.

Extensive literatures have indicated that ROS plays a crucial role in cell apoptosis and participates in multiple signaling pathways which can mediate high anti-proliferation effect.[29] In order to determine the pathway by which Que, IS and I3G induced apoptosis, we examined ROS generation in MCF-7 cells.[30,31] Our results showed that Que, IS and I3G led to a significant dose-dependent increase of intracellular ROS in MCF-7 cells, and the antioxidant effect order is as follows: Que > IS > I3G, indicating that ROS production led to apoptotic cell-death through the mitochondrial pathway (Fig. 5). In agreement with some previews report, Que, IS and I3G possess strong antioxidation activities *in vitro*.[32,33] The result of "Que > IS" is in agreement with a previous study which revealed that radical scavenging activity decreased

295 in the order Que > IS.[34] The result of "IS > I3G" has never been compared before, and this may
296 related to the fact that in quercetin and its derivatives, 3-OH is an important activity position,
297 glucuronidation atthe 3-postion had a marked decrease in their antioxidant activity.[35] The assay
298 results of H_2O_2 and O_2^- indicated that ROS played a crucial role in cell apoptosis, and ROS
299 might participates in the Que-, IS- and I3G-elicited MCF-7 cell death. In the subsequent study,
300 we will explore the relationship of ROS and Que, IS and I3G induced MCF-7 cell death by
301 using the antioxidant such as Acetylcysteine (NAC), DPI and so on. Overall, we firstly reveals
302 the reality that IS and I3G, which are main metabolites of quercetin *in vivo*, possess strong
303 tumor-inhibitory activities in MCF-7 cells via cell circle arrest at S phase and apoptosis by
304 ROS-dependent mitochondrial pathway, indicating that quercetin metabolites may still possess
305 strong activities *in vivo*.

306 In addition, the anti-tumor mechanism of Que, IS and I3G may also be related to the
307 cell-permeability of them. The extensive reports have indicated that quercetin has the high
308 cell-permeability,[9] which may contribute to its cytotoxic activity. An extensive literatures have
309 indicated that quercetin has a significantly high anti-proliferation effect, which may also
310 contribute to its antitumor activity *in vitro*.[36] As to the "Que > IS> I3G", we think that it may
311 be related to the different molecular weight and polarity of them. Small molecules was easier to
312 across cell membrane and the molecular weight of these compounds was sorted as Que
313 (302.24) > IS (316.2623) > I3G (492.39), which was consistent with the cytotoxic efficiency
314 order of them.

315 Meanwhile, we investigated the growth-inhibitory effects and cytotoxicity of Que, IS and
316 I3G on normal mammary epithelial cell H184B5F5/M10 cell. As shown in Fig. 6, Que, IS and

317 I3G has no cytotoxicity against the normal mammary epithelial cells, indicating that Que, IS
318 and I3G have a good selectivity on the tested tumor cells. Our previous studies have showed
319 that Que and QS (5', 8-disulfonate substituted metabolite of quercetin) possess high anti-tumor
320 activity in human colon cancer LoVo cells and breast cancer MCF-7 cells,[11] which can further
321 provide evidence for the application of the tested Que, IS and I3G. Furthermore, some other
322 studies have showed that Que has strong anticancer effect on a wide range of cancer cells such
323 as acute lymphoid, myeloid leukemia cells, human gastric and colon cancer cells.[7-11] It is also
324 reported that Que, IS and I3G have high antioxidant ability,[34] which further provide evidence
325 for the application of the three compounds. In general, after Que ingestion, its metabolites
326 quercetin, isorhamnetin and isorhamnetin-3-glucuronide are mainly present in the
327 physiological fluids, and concentrations of animal tissues are in the range of 0.015-0.125 uM,
328 0.53-0.65 uM or 0.03-0.18 uM.[23, 37] It must be noted that the concentrations (25, 50 and 100
329 μM) we used in this study may generally be not physiological and achievable *in vivo* because
330 of the low bioavailability of these compounds. In this study we mainly investigated the
331 anticancer-structure relationships between Que, IS and I3G in vitro and try to provide the
332 foundation for high bioavailability and water solubility metabolites of quercetin in vivo
333 evaluation, and to evaluate the potential clinical use of this study.

334 In conclusion, we investigated and compared the cytotoxic activities of Que, IS and I3G in
335 human breast cancer MCF-7 cells for the first time, and found that they possess strong
336 cytotoxic effect through a ROS-dependent apoptosis pathway in MCF-7 cells. Significantly, we
337 firstly point out the fact that 3'-methylation can decrease the cytotoxic properties of querctein,
338 and 3-glucuronidation may further decrease the cytotoxic activity of IS (3'-methylation of

quercetin), and display the structure-anti tumor activity relationship of them (Que > IS > I3G). In our previous studies, we have investigated and compared the cytotoxic effect of Que and its another metabolite Q3G (glucuronidation metabolite of quercetin in 3 position) on human breast cancer MCF-7 cells, the results showed that Que and Q3G possess cytotoxic effect in breast cancer MCF-7 cells and it was ordered "Que > Q3G", indicating that glucuronidation may decreased the cytotoxic effect of Que *in vitro*.[38] Our studies *in vitro* provide a new insight into the cytotoxic effect of quercetin metabolites, and further studies should be carried out in animal studies and ultimately in clinical trials.

In the subsequent study, we will further develop the synthesis method and do some metabonomics experiments to further investigate the cytotoxic mechanism of Que, IS and I3G in MCF-7 cells. Metabolomics is an integral part of the systems biology and is rapidly advancing with the aims of detecting many metabolites with low molecular weight in single cell, bio-fluids, and tissue extracts. [39] Therefore, the application of metabolomics method may contribute to the further understanding of the cytotoxic mechanism of Que, IS and I3G.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interest.

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Figure Captions

Fig.1. Chemical structure of quercetin (Que), Isorhamnetin (IS) and Isorhamnetin-3-glucuronide (I3G)

Fig.2. The MTT assay results of Que, IS and I3G on MCF-7 cells was assayed (A), and cytotoxic effect was measured by LDH assay (B) and the change of cell morphology was measured by optic microscopic observation (C). In Fig 2(C), red arrow means normal MCF-7 cells, yellow arrow means cell shrinkage, green arrow means cell fusion and bronzing arrow means cell lysis. Data are expressed as the mean ± SD (n = 3). $p<0.05$ (*) or $p<0.01$ (**) indicates a significant difference versus control.

Fig.3. Quantitative analysis of apoptotic cells induced by Que, IS and I3G using annexin V/PI double staining assay. (A) Representative dot plots of Annexin V/PI staining. (B) Column bar graph of apoptotic cells. Cells were treated with Que, IS and I3G at 25 and 100 μM for 48 h, respectively. 3000 cells were analyzed by flow cytometry. The results are expressed as mean ± SD of three independent experiments. $p<0.01$(**), as compared to the control.

Fig.4. Effects of Que, IS and I3G on cell circle phase distribution of MCF-7 cells. (A) Representative histograms of DNA content in the cells incubated with Que, IS and I3G at 50 and 100 μM for 48 h. Horizontal and vertical axes indicate the relative nuclear DNA content and number of cells, respectively. (B) Percentage of cell populations in G0/G1, S and G2/M phases. All values are expressed as mean ± SD of three independent experiments. Significant

489 difference from the control at the same phase is indicated at $p<0.05$ (*) or $p<0.01$ (**).

490

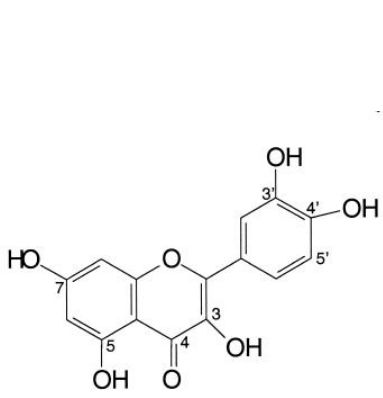
491 **Fig.5.** Effects of Que, IS and I3G on ROS (H_2O_2 and O_2^-) generation of MCF-7 cells. (A)
492 Representative flow cytometric images for O_2^- generation in MCF-7 cells. (B) Levels of O_2^- (%)
493 in MCF-7 cells when treated with Que, IS and I3G. (C) Fluorescence intensity analysis for
494 H_2O_2 generation in MCF-7 cells. (D) Levels of H_2O_2 (%) in MCF-7 cells when treated with
495 Que, IS and I3G. About 100 μM H_2O_2 were used as positive control. The results represent the
496 mean ± SD of three independent experiments. $p<0.05$ (*) or $p<0.01$ (**) indicate statistically
497 significant difference with control, which was considered to be 100%.

498

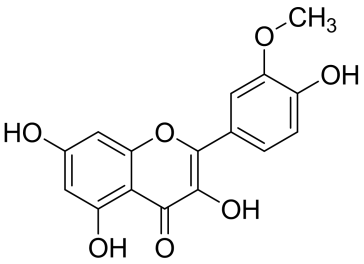
499

500 **Fig.6.** Growth-inhibitory effects and cytotoxicity of Que, IS and I3G on normal mammary
501 epithelial H184B5F5/M10 cells. MTT assay (A) and LDH assay (B). The results represent the
502 mean ± SD of three independent experiments. $p<0.05$ (*) or $p<0.01$ (**) indicate statistically
503 significant difference with control, which was considered to be 100%.

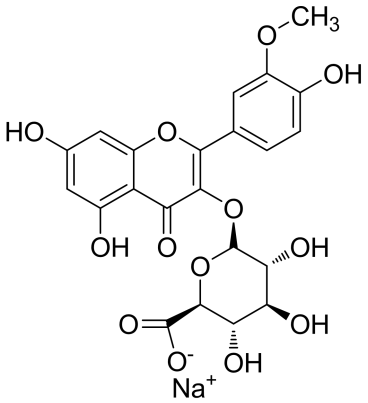
Figure 1



Que



IS



I3G

Figure 2

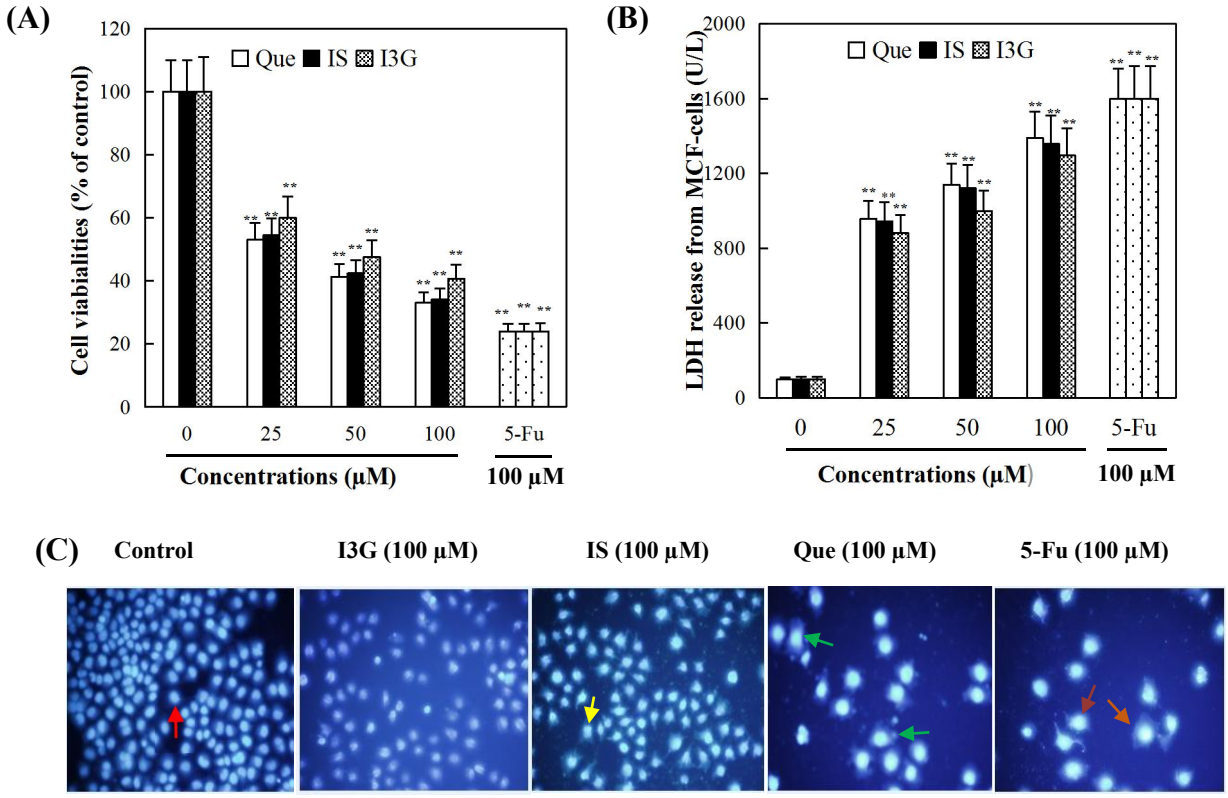
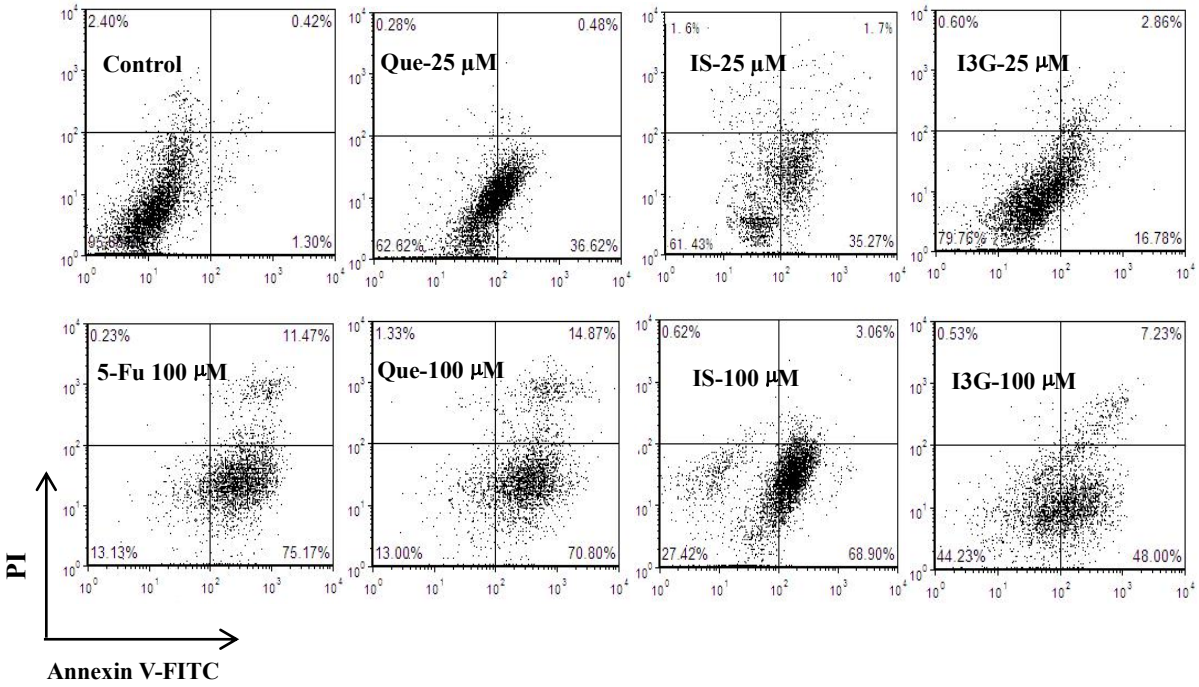

(A)
Cell viabialities (% of control)
120
100
80
60
40
20
0
Que IS I3G
0
25
50
100
5-Fu
Concentrations (μM)
100 μM
(B)
LDH release from MCF-cells (U/L)
2000
1600
1200
800
400
0
Que IS I3G
0
25
50
100
5-Fu
Concentrations (μM)
100 μM
(C)
Control
I3G (100 μM)
IS (100 μM)
Que (100 μM)
5-Fu (100 μM)

Figure 3

(A)


Control
2.40%
0.42%
1.30%
Que-25 μM
0.28%
0.48%
62.62%
36.62%
IS-25 μM
1.6%
1.7%
61.43%
35.27%
I3G-25 μM
0.60%
2.86%
79.76%
16.78%
5-Fu 100 μM
0.23%
11.47%
13.13%
75.17%
Que-100 μM
1.33%
14.87%
13.00%
70.80%
IS-100 μM
0.62%
3.06%
27.42%
68.90%
I3G-100 μM
0.53%
7.23%
44.23%
48.00%
PI
Annexin V-FITC

(B)

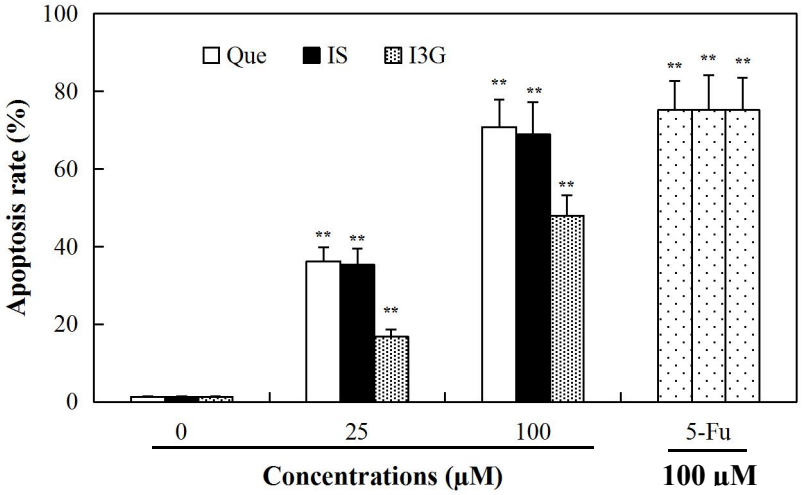
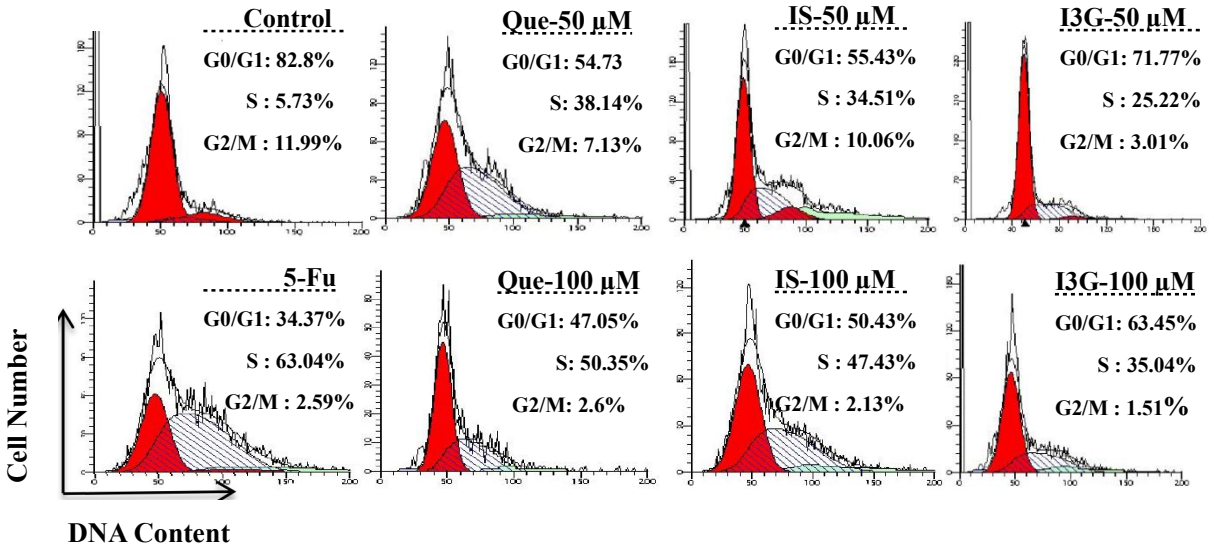

Que
IS
I3G
Apoptosis rate (%)
100
80
60
40
20
0
0
25
100
5-Fu
Concentrations (μM)
100 μM

Figure 4

(A)


Control
G0/G1: 82.8%
S : 5.73%
G2/M : 11.99%
Que-50 µM
G0/G1: 54.73
S: 38.14%
G2/M: 7.13%
IS-50 µM
G0/G1: 55.43%
S : 34.51%
G2/M : 10.06%
I3G-50 µM
G0/G1: 71.77%
S : 25.22%
G2/M : 3.01%
5-Fu
G0/G1: 34.37%
S : 63.04%
G2/M : 2.59%
Que-100 µM
G0/G1: 47.05%
S: 50.35%
G2/M: 2.6%
IS-100 µM
G0/G1: 50.43%
S : 47.43%
G2/M : 2.13%
I3G-100 µM
G0/G1: 63.45%
S : 35.04%
G2/M : 1.51%
Cell Number
DNA Content

(B)

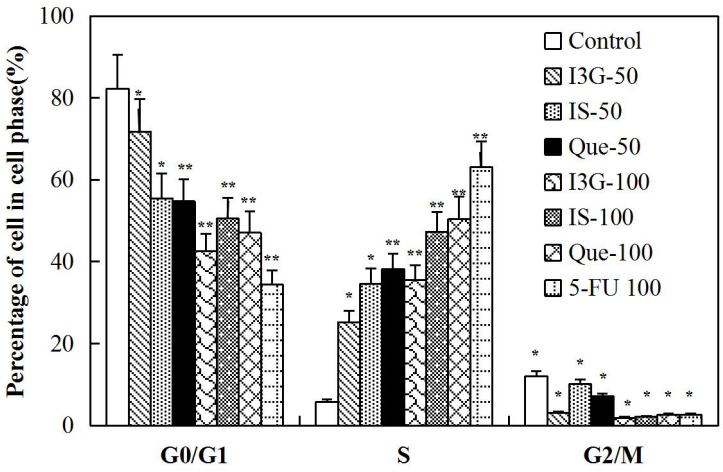

Percentage of cell in cell phase(%)
100
80
60
40
20
0
G0/G1
S
G2/M
Control
I3G-50
IS-50
Que-50
I3G-100
IS-100
Que-100
5-FU 100

Figure 5

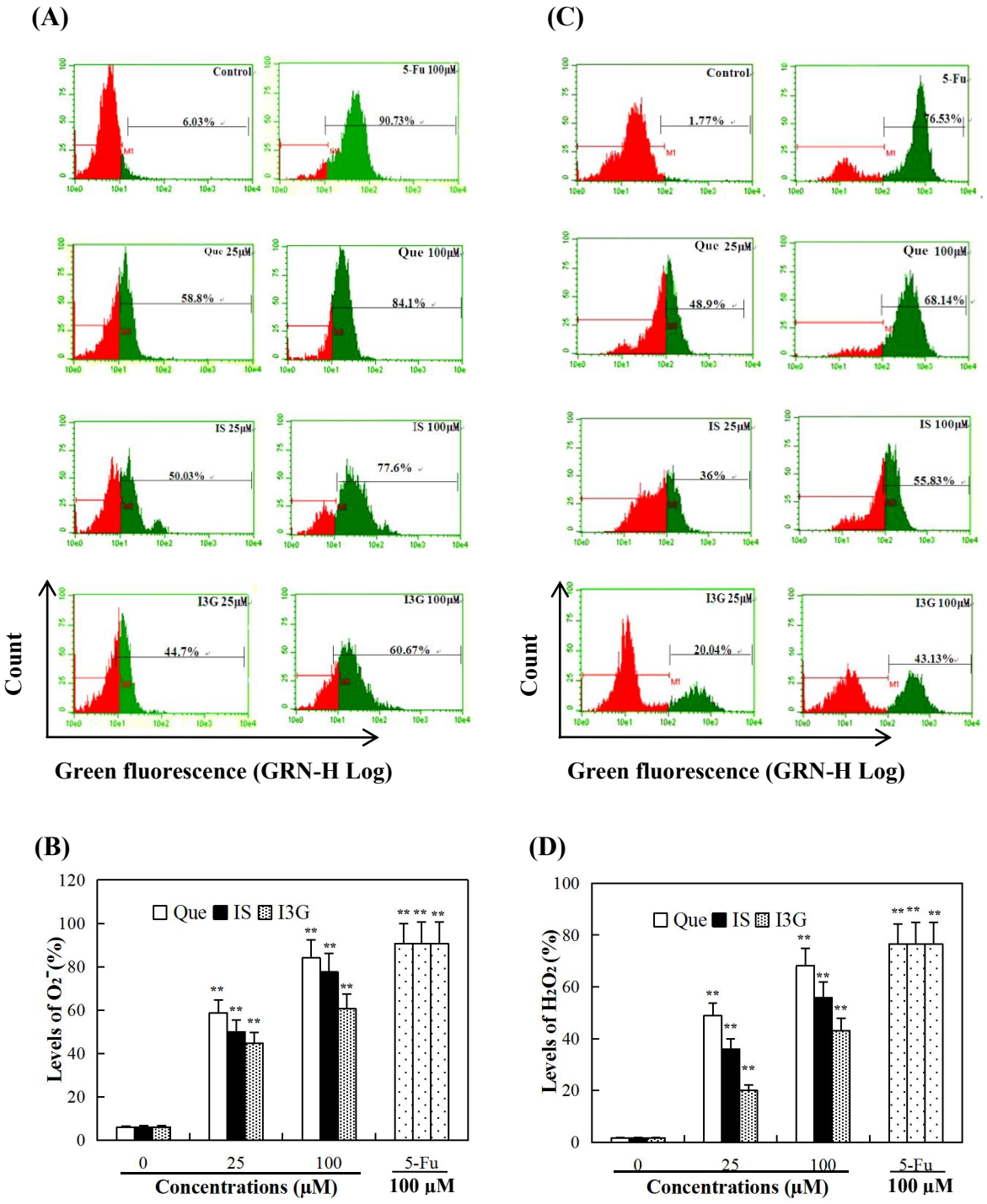
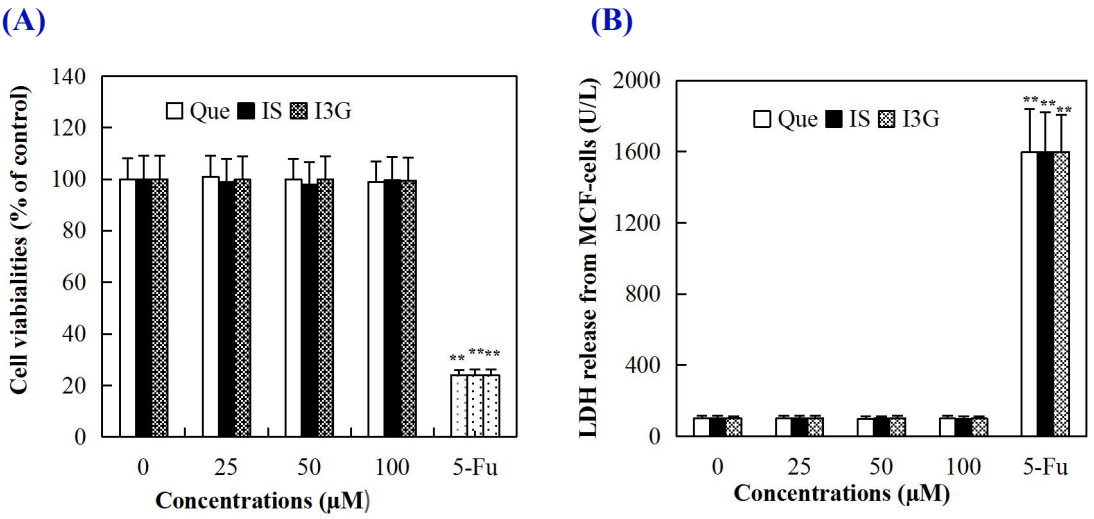
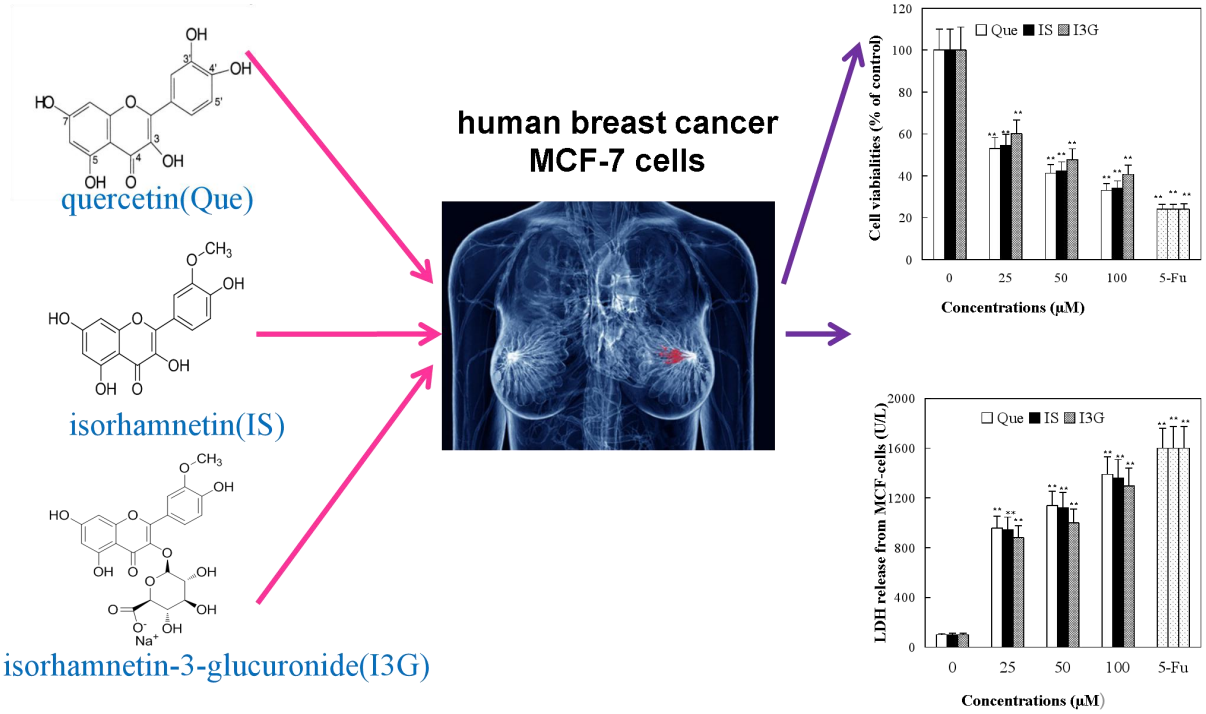
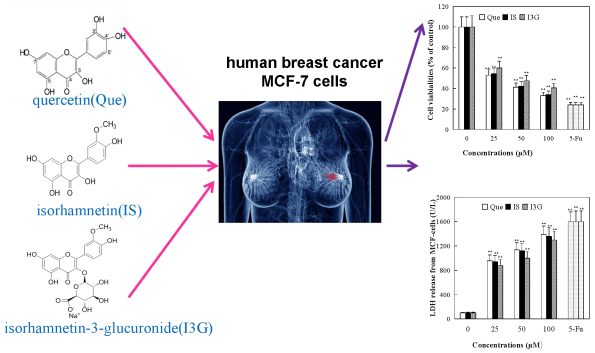

(A)
Control
6.03%
5-Fu 100μM
90.73%
Que 25μM
58.8%
Que 100μM
84.1%
IS 25μM
50.03%
IS 100μM
77.6%
I3G 25μM
44.7%
I3G 100μM
60.67%
Count
Green fluorescence (GRN-H Log)
(C)
Control
1.77%
5-Fu
76.53%
Que 25μM
48.9%
Que 100μM
68.14%
IS 25μM
36%
IS 100μM
55.83%
I3G 25μM
20.04%
I3G 100μM
43.13%
Count
Green fluorescence (GRN-H Log)
(B)
Que IS I3G
Levels of O2- (%)
0 25 100 5-Fu
Concentrations (μM)
100 μM
(D)
Que IS I3G
Levels of H2O2 (%)
0 25 100 5-Fu
Concentrations (μM)
100 μM

Figure 6


(A)
(B)
Que IS I3G
Cell viabilities (% of control)
LDH release from MCF-cells (U/L)
Concentrations (μM)
0
25
50
100
5-Fu
140
120
100
80
60
40
20
0
2000
1600
1200
800
400
**



TOC Graphic (original drawing)



TOC Graphic(shrunken drawing)