Effect of Methoxy flavones on proliferation, cell cycle progression and apoptosis of human hepatocellular carcinoma (HepG2) cells

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ABSTRACT

Hepatocellular carcinoma (HCC) is the fifth most common forms of cancer occurring worldwide and its incidence is on the rise. The treatment options available currently have certain limitations and research is on for discovering new molecules with a better outcome profile. Bioactive compounds from plants have shown promise in the treatment of HCC. Flavonoids are plant secondary metabolites and diets rich in these have been shown to decrease the incidence of various forms of cancer. Several studies have indicated the anticancer ability of various flavonoids to act by various mechanisms. In the present study three dimethoxy flavones viz., 3,6-dimethoxy flavone (3,6-DMF), 6,2’-dimethoxy flavone (6,2’-DMF) and 6,3’-dimethoxy flavone (6,3’-DMF) were evaluated for anti proliferative activity and apoptosis inducing ability against HepG2 cell line. All the three compounds have shown dose dependent anti proliferative activity at the doses of 50, 100, 150, 200 and 250 µg/ml and the IC50 values were 200 µg/ml for 3,6-DMF and 6,2’-DMF and for 6,3’-DMF the value was 150 µg/ml at 48h. The compounds were subjected to flow cytometry and it was seen that the three compounds caused an increase in the number of cells in the G1 phase. All the 3 compounds were shown to promote apoptosis as was seen in the nuclear morphological examination study using Propidium iodide staining and DNA fragmentation by the agar gel electrophoresis technique. Our study has shown that the three dihydroxy flavones may hold promise for development as a drug of use in the treatment of Hepatocellular carcinoma.

Key words: Apoptosis, Antiproliferative, Flavonoids, Flow cytometer, 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-dimethyltetrazolium bromide (MTT), Propidium iodide

INTRODUCTION

Hepatocellular carcinoma (HCC) is the primary form of human adult liver cancer and it is the fifth most commonly occurring forms of cancer worldwide. It is known to occur widely in China, most parts of South East Asia and South Africa. The incidence of HCC is on the rise with one million new cases being diagnosed every year and an equal number of deaths occurring. Some of the options available for treatment are orthotopic liver transplantation, surgical resection and local destruction. The recurrent rate continues to be high with 50% recurrence in two years. The other option available is chemotherapy and treatment with interferons and hormones. This also remains a challenge as HCC is highly resistant to systemic treatments. The search for a more active and specific treatment for liver cancer is continuing and many molecular compounds derived from plants have shown promise.

Flavonoids are secondary metabolites present in plants. Dietary flavonoids and other polyphenols have been demonstrated to have cancer preventive properties in various biological systems. Many studies suggest that high flavonoid intake is associated with decreased risk of cancer. Prevention of carcinogen metabolic activation, inhibition of angiogenesis, antioxidative activity, promotion of differentiation, induction of apoptosis, cell cycle arrest and antiproliferation are some of the mechanisms by which flavonoids exhibit their anticancer activity. Silybin, a polyphenolic compound has shown antiproliferative activity against many cancer cell lines including Hepatocellular carcinoma (HCC). It causes G1 arrest in HepG2 and G2 and G2-M arrests in Hep3B cells. Quercetin causes cell cycle arrest such as G2/M arrest or G0 arrest. The present study is an attempt to explore the anti proliferative and apoptotic ability of a few synthetic dimethoxy flavones on HepG2 cell lines. The flavones tested were 3,6- dimethoxy flavone (3,6-DMF), 6,2’-dimethoxy flavone (6,2’-DMF) and 6,3’-dimethoxy flavone (6,3’-DMF). These compounds have been shown to possess antioxidant and hepatoprotective activity in our previous study.

MATERIALS AND METHODS

1. Chemicals

The Rosewell Park Memorial Institute (RPMI)-1640 medium, Fetal Bovine Serum (FBS), 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-dimethyltetrazolium bromide (MTT), Propidium iodide were purchased from Sigma chemicals (St Louis, MO, USA). All the chemicals used were of analytical grade. The compounds used for the study 3,6-dimethoxy flavone (3,6-DMF), 6,2’-dimethoxy flavone (6,2’-DMF) and 6,3’-dimethoxy flavone (6,3’-DMF) were obtained from Research Organics Ltd, Chennai- 41. The compounds were dissolved in dimethyl formamide to prepare stock solutions.

2. Cell lines and cell cultures

HepG2 (Human hepatic carcinoma cell line) was obtained from the National Centre for Cell Sciences (NCCS), Pune, India. The cells were cultured in RPMI supplemented with 10% v/v FBS, penicillin 100µg/ml, Streptomycin 20µg/ml, Kanamycin acid sulphate 20µg/ml and 7.5% Sodium bicarbonate solution. The cells were maintained as monolayers in 25cm2 tissue culture flasks at 37°C in a humidified atmosphere containing 5% CO2. Exponentially growing cells were used in all the experiments.

a. In vitro assay for cytotoxic activity (MTT assay)

The cytotoxicity of dimethoxy flavones on both MCF-7 cells was determined by the MTT assay. HepG2 cells (5x103 cells/ml) were plated in 96-well plates with RPMI-1640 medium containing 10% FBS. The cells were incubated for 24h under 5% CO2 & 95% O2 at 37°C. The medium was removed and washed with PBS and then fresh medium was added and kept for 4h in the incubator. The medium was removed and fresh medium was added to control wells and 50, 100, 150, 200 and 250 µg/ml of test drug solution containing medium was added to the treatment plates. The culture plates were incubated as above. After 24, 48 and 72h, 10µ1 of 5mg/ml MTT solution was added to each well and the cultures were further incubated for 4h and then 100µl DMSO was added and the formed crystals were dissolved gently by pipetting 2 or 3 times. A micro plate reader was used to.
measure absorbance at 570nm for each well. Cell viability was calculated using the formula:

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\text{Cell viability} = \left( \frac{A_t}{A_s} \right) \times 100
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where \(A_t\) is absorbance in test and \(A_s\) is the absorbance in standard.

b. Nuclear morphological examination
Cancer cells (5x10^3 cells/ml) were seeded in six well-plates with RPMI-1640 medium containing 10% FBS. The cells were incubated for 24h under 5% CO\(_2\), 95% O\(_2\), at 37°C. Then the medium was removed and the control wells received again fresh medium and the treatment plates received 200µg/ml concentrations of test drug solutions. Then the culture plates incubated as above for 24, 48 and 72h. After completion of incubation time the cells were washed with PBS and fixed in methanol: acetic acid (3:1) for 10min and stained with 4µg/ml of propidium iodide for 20min. After staining the cells were visualized immediately under Confocal Laser Scanning Microscopy (LSM) at 20X magnification.

c. Cell cycle analysis
For cell cycle analysis, cells were seeded in six well plates with RPMI-1640 medium containing 10% FBS. The cells were incubated for 12h under 5% CO\(_2\), 95% O\(_2\), at 37°C. Then, the RPMI-1640 with FBS was removed and washed with PBS. Fresh medium was added and kept for 1h in the incubator. The medium was removed and fresh medium was added to the control wells. The treatment plates received appropriate concentrations of test solution containing drug for 48h. Then, the RPMI-1640 with FBS was removed and washed with PBS. The cells were incubated for 12h under 5% CO\(_2\) & 95% O\(_2\) at 37°C. To this, 4µl of propidium iodide stain was added, mixed and centrifuged at 500 rpm for 15min to separate the DNA. Phenol-chloroform extraction was repeated twice, followed by chloroform extraction alone. To the resulting aqueous phase, 2 volumes of ice-cold absolute ethanol and 0.1 volume of 3M sodium acetate were added and incubated for 30min on ice to precipitate DNA. DNA was pelleted by centrifuging at 13,000 rpm for 10 min at 4°C, the supernatant was aspirated and the pellet was washed with 1ml of 70% ethanol. After repeating the above centrifugation step and removing the last traces of the supernatant fraction, the pellet was allowed to dry at room temperature for approximately 30min and resuspended in 50µl of TE buffer. The DNA was quantified by UV-visible spectroscopy and 10µg of DNA was electrophoresed in a 1% agarose gel containing ethidium bromide in a mini gel tank containing TBE buffer for 1h under 9V. Then the gel was examined under UV transilluminator (Biorad) and photographed.

d. DNA-fragmentation analysis by agarose gel electrophoresis
DNA preparation an agarose gel electrophoresis was carried out according to a method previously reported. Cancer cells (3x10^3 cells/ml) were plated per well in six well plates with RPMI-1640 medium containing 10% FBS. The cells were incubated for 12h under 5% CO\(_2\), 95% O\(_2\), at 37°C. Then, the RPMI-1640 with FBS was removed and washed with PBS. Fresh medium was added and kept for 1h in the incubator. The medium was removed and fresh medium was added to the control wells. The treatment plates received appropriate concentrations of test solution containing drug for 48h. Then, the RPMI-1640 with FBS was removed and washed with PBS. The cells were incubated for 12h under 5% CO\(_2\) & 95% O\(_2\) at 37°C. To this, 4µl of propidium iodide stain was added, mixed and centrifuged at 500 rpm for 15min to separate the DNA. Phenol-chloroform extraction was repeated twice, followed by chloroform extraction alone. To the resulting aqueous phase, 2 volumes of ice-cold absolute ethanol and 0.1 volume of 3M sodium acetate were added and incubated for 30min on ice to precipitate DNA. DNA was pelleted by centrifuging at 13,000 rpm for 10 min at 4°C, the supernatant was aspirated and the pellet was washed with 1ml of 70% ethanol. After repeating the above centrifugation step and removing the last traces of the supernatant fraction, the pellet was allowed to dry at room temperature for approximately 30min and resuspended in 50µl of TE buffer. The DNA was quantified by UV-visible spectroscopy and 10µg of DNA was electrophoresed in a 1% agarose gel containing ethidium bromide in a mini gel tank containing TBE buffer for 1h under 9V. Then the gel was examined under UV transilluminator (Biorad) and photographed.

RESULTS

a. Cell viability assay against HepG2 cancer cell lines
The RPMI-1640 medium containing 10% FBS was removed and washed with PBS. The cells were incubated for 24h under 5% CO\(_2\), 95% O\(_2\), at 37°C. Then the medium was removed and the control wells received again fresh medium and the treatment plates received 200µg/ml concentrations of test drug solutions. Then the culture plates incubated as above for 24, 48 and 72h. After completion of incubation time the cells were washed with PBS and fixed in methanol: acetic acid (3:1) for 10min and stained with 4µg/ml of propidium iodide for 20min. After staining the cells were visualized immediately under Confocal Laser Scanning Microscopy (LSM) at 20X magnification.

b. Nuclear morphological examination
To confirm whether the cytoxic effect induced by 3, 6-DMF, 6, 2'-DMF and 6, 3'-DMF involves apoptosis changes, the nuclear condensation was studied by the propidium iodide staining method. In the case of control cells, a very negligible number of propidium iodide positive cells were present. In the case of cells treated with 200µg/ml of 3, 6-DMF and 6, 2'-DMF and 150µg/ml of 6, 3'-DMF and with exposure times of 24h, 48h and 72h, a progressive increase in the number of propidium iodide positive cells was observed (Fig.2).

c. Cell cycle analysis
The HepG2 cells were incubated with 200µg/ml of 3, 6-DMF and 6, 2'-DMF and 150µg/ml of 6, 3'-DMF for 48h and then were subjected to cell cycle analysis. In the control, the percentage of cells in the different phases
of cell cycle were 10.14% in G\textsubscript{1} phase, 50.3% in G\textsubscript{1}/S phase, 13.14% in the S phase and 26.77% in the G\textsubscript{2} phase. Cells treated with 3, 6-DMF for a period of 48h, showed 87.5% cells in G\textsubscript{1} phase, 10.54% cells in G\textsubscript{1}/S phase, 1.02% in the S phase and 0.99% in the G\textsubscript{2} phase. 6, 2’-DMF treatment also produced a similar pattern of cell distribution with 78.19% cells in G\textsubscript{1} phase, 14.54% cells in G\textsubscript{1}/S phase, 2.72% in the S phase and 4.99% in the G\textsubscript{2} phase. With 6, 3’-DMF treatment also, a marked increase in cells was seen in the G\textsubscript{1} phase. The cells in the G\textsubscript{1}, G\textsubscript{1}/S, S and G\textsubscript{2} phase were 94.1%, 5.21%, 0.42% and 0.35% respectively (Fig.3).

d.DNA fragmentation analysis
DNA fragmentation analysis is a typical assay to analyse drug induced apoptotic cell death. The cells were treated with 200µg/ml of 3, 6-DMF and 6, 2’-DMF and 150µg/ml of 6, 3’-DMF for a period of 48h. In the control lane the DNA was intact without any fragmentation. In the lanes which received treated cells, there was DNA fragmentation which is seen as a “ladder pattern” (Fig.4).

DISCUSSION
A number of flavonoids have exhibited antineoplastic activity\textsuperscript{[13]}. Epidemiological studies suggest that a reduced risk of cancer is associated with consumption of diet rich in phytochemicals\textsuperscript{[14]}. Fruits and food products contain high levels of a diverse range of phytochemicals of which flavonoids make up a large proportion\textsuperscript{[15-17]}. The fact that phytochemicals reduce the risk of cancer is supported by a number of \textit{in vitro} and \textit{in vivo} studies which reveal that flavonoids may act at the various stages involved in carcinogenic processes such as carcinogen inactivation, antiproliferation, cell cycle arrest, induction of apoptosis and differentiation and inhibition of angiogenesis\textsuperscript{[18]}. Dietary flavonoids are natural antioxidants\textsuperscript{[19]}. They may act against cancer by limiting the damaging oxidative reactions in cells, which may be a predisposing factor for the development of cancer. The oxygen derived free radicals seem to have the ability to initiate as well as promote carcinogenesis. The lipid peroxidation products originating from dying cells could also exert a cancer promotional effect\textsuperscript{[20, 21]}. Tissue culture tests, the \textit{in vitro} anticancer activity screening tests, are used to evaluate potential anticancer agents\textsuperscript{[22]}. In the present study also, the \textit{in vitro} cell viability assay against HepG2 cancer cell line was used to evaluate the growth inhibition potential of the test compounds. The compounds produced cytotoxicity and also showed a growth inhibition activity in a dose and time dependant manner. The viability of the cells was assessed by the MTT assay and the cytotoxic nature of the cells was confirmed by changes in the morphological character of the cells. At the end of 48h, 50% viability was seen with a concentration of 200µg/ml for 3,6-DMF and 6,2’-DMF whereas for 6,3’-DMF it was 150µg/ml. The decrease in viable cells at the end of 48h and 72h indicates antiproliferative activity.

Alterations in cell cycle progression may also account for the anticarcinogenic effect of flavonoids. Synthesis of DNA (S phase) and separation of two daughter cells (M phase) are the main features of cell cycle progression. The time between S and M phase is the G\textsubscript{2} phase. G\textsubscript{1} phase represents the period of commitment to cell cycle progression that separates M and S.
phases, as cells prepare for DNA duplication on receiving mitogenic signals. Check points at both G_{1}/S and G_{1}/M of the cell cycle in cultured cancer cell lines have been found to be altered by flavonoids such as silymarin, quercetin, luteolin and apigenin\textsuperscript{[23-28]}. The test compounds were also subjected to cell cycle analysis. The dose chosen was the approximate IC_{50} dose at the 48th time of exposure. With all the compounds, there was a significant increase in the number of cells in the G_{1} phase. It has been shown that flavopiridol could induce a similar cell cycle arrest in the G_{1} phase by the inhibition of CDKs. These have been recognized as key regulators in cell cycle progression.

Apoptosis is another mechanism by which flavonoids produce significant anticancer property \textsuperscript{[29-31]}. Apoptosis is an active form of cell death that plays an important role in development and survival by eliminating any unwanted or damaged cells. Dysregulation of apoptosis plays a critical role in oncogenesis. Many chemotherapeutic agents exert their tumoricidal effects by inducing apoptosis in target cells and tissues. Flavonoids have also been shown to induce apoptosis in some cancer cell lines \textsuperscript{[32]}

In the present study, the ability of the compounds to induce apoptosis was evaluated using propidium iodide staining method. One of the characteristics of cells undergoing apoptosis is nuclear chromatin condensation. The DNA in condensed chromatin stains strongly with fluorescent dyes which allows for differentiation of apoptotic from non apoptotic cells \textsuperscript{[33]}. The apoptotic cells can be differentiated from non apoptotic cells as the former are bright and their nuclei condensed. The results of the present study also showed an increase in apoptosis as was seen with an increase in the number of cells with stained nuclear chromatin with all the three compounds.

DNA fragmentation is a secondary consequence, rather than an integral cause of apoptosis. DNA fragmentation is analysed using agarose gel electrophoresis which shows a classic ladder pattern. Necrosis, on the other hand, is characterised by random DNA fragmentation which forms a “smear” on agarose gel. In the present study too, the three compounds showed a ladder pattern indicating apoptosis.

Suggested mechanism of anticancer effects of polyphenols includes antioxidant, anti-inflammatory and antiproliferative effects as well as their effects on subcellular signalling pathways, induction of cell cycle arrest and apoptosis \textsuperscript{[34]}. Hence, it is possible that the three dimethoxy flavones would have the potential to be useful anticancer agents because they have been shown to be antiproliferative agents with apoptosis inducing ability along with antioxidant properties.

**REFERENCE**

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