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RESEARCH ARTICLE

In Vitro Anti-Cancer Activity of Quercetin and Kaempferol against Human Epithelial Malignant Melanoma Cells (A375)

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ABSTRACT

In the present study, in vitro anti-cancer activity of two flavonoids, quercetin and Kaempferol, was studied against human epithelial malignant melanoma cells (A375). Both the compounds were subjected to cytotoxicity assay. Quercetin and Kaempferol were investigated for their effects on apoptotic gene such as caspase3, bax, p53 and anti-apoptotic gene Bcl2 expression in A375 Cell lines by reverse transcriptase Polymerase Chain Reaction. MTT assays reveals that the IC50 value of quercetin and Kaempferol was found to be 1.54 and 12.05 ng/ml in the tested condition. Both the flavonoids down-regulated caspase3, bax, p53 and up-regulated Bcl2 expression. Results of the present study reveals that both the compounds may be interesting candidates for further studies on the molecular mechanism of action in skin carcinoma.

KEYWORDS

A375 Cell Lines, Quercetin, Kaempferol, Anti-Cancer, Apoptosis, mRNA Expression

INTRODUCTION

Worldwide the incidences of melanoma and non-melanoma skin cancer is showed to be increasing than other forms of the cancers[1,2] due to various risk factors like excess exposure to ultraviolet B radiation, latitude, climatic conditions, environmental pollutants, occupational carcinogens, active / passive smoking, ageing, family history etc. On the other hand, the depletion of stratospheric ozone is implicated as one of the major risk factor for skin carcinoma[3]. In India, though no clear data available on the prevalence / incidence of skin cancer in Indian population, but indirect surveys indicate that non-melanoma skin cancers (NMSCs) may be on the rise in India[4]. Melanoma cells develop resistance to chemotherapeutics very rapidly and thus complicates the treatment. The search for newer therapeutics is more important for effective treatment / management of skin cancer. Plant based drugs are being used for chemoprevention and also to suppress the malignancy of cancer. Flavonoids are one of the major secondary metabolites obtained from plant sources which exhibit broad beneficial effects in human health. Quercetin (3, 3', 4', 5, 7- pentahydroxylflavone) (Figure 1), found in abundant in vegetables and fruits. Quercetin reported to prevent the oxidation of low-density lipoproteins (LDL) by scavenging free radicals. It also reported to impart beneficial effects in the treatment of cancer, chronic inflammation and atherosclerosis[5,6].

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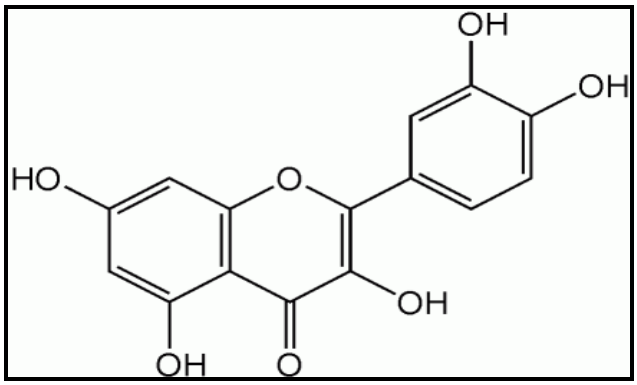

Figure 1: Quercetin

Kaempferol, (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) (Figure 2) is one of the commonly found flavonoids in plant based foods and herbs used in traditional medicines. Several reports demonstrate the possible association and consumption of diet rich in kaempferol and decreased risks of several diseases like cancer, cardiovascular, neurological and ageing[7].

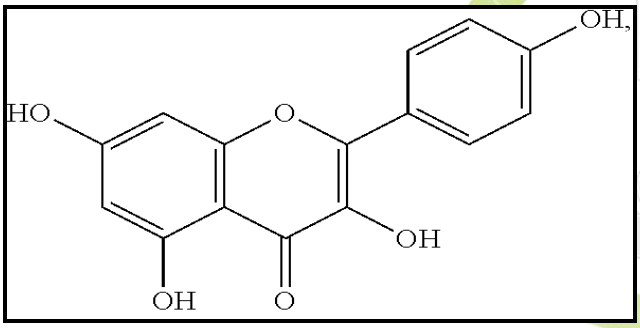

Figure 2: Kaempferol

The present study was undertaken to evaluate the inhibitory effects of quercetin and kaempferol on the cell proliferation and their ability to trigger apoptosis in human epithelial malignant melanoma cells (A375). Both the chemical principles triggered A375 cell death via apoptotic pathway.

MATERIAL AND METHODS

Chemicals and Reagents

Quercetin, kaempferol and TRIzol reagent were procured from Sigma-Aldrich, US. Dulbecco's modified Eagle's medium (DMEM) and serum (fetal bovine serum) were obtained from GIBCO-BRL (Gaithersburg, MD, USA). MTT was procured from Himedia, India. Primers and one step master mix procured from Biogene, India. All other chemicals and reagents used were of analytical grade, unless specified.

Cell Culture and Maintenance

A375, a human epithelial malignant melanoma cells were obtained from National Centre for Cell Science (NCCS), Pune, India. Cells were maintained in Dulbecco's Modified Eagle's Medium - high glucose (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), with 100units/ml penicillin and 100µg/ml streptomycin. Cells were cultured in 75cm^2 culture flask and incubated at humidified atmosphere with 5% CO_2 at 37°C.

MTT Assay[8]

Cell respiration is an indicator of cell viability and proliferation which usually is determined using a mitochondrial dependent reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5- Diphenyl tetrazolium bromide (MTT) to formazan. Preconfluent A375 cells were seeded in 96-well plates at a density of 8,000 cells/well. Cells were treated with different concentrations of the test drug (ranging from 1X10^3 - 1X10^6 ng) after 24 h following plating and incubated at 37°C for one day. At 20 h following drug exposure, the cells were incubated at 37°C with 0.5 mg/ml MTT for 4 h. At the end of the experiment, the medium was removed, and the insoluble formazan product was dissolved in DMSO (200µl) and kept at least 15 min in dark. The intensity of purple blue colour developed was measured at 570 and 630 nm using Thermoscientific multiscan spectrophotometer, USA. Percentage growth inhibitory rate of the test drug was calculated using the formula

% Growth Inhibitory Rate = ([Control OD – Test OD] / Control OD) * 100

Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR)[9]

A375 cells were seeded in 6 well plates at a density of 1X10^5 cells/well and allowed to grow for a period of 24 h. Test drug was administered at 3 different concentration (Quercetin: 0.1, 1 and 10ng/ml and Kaempferol: 1, 10 and 100 ng/ml).

Cells were then trypsinised for measuring gene expressions of bax, bcl2, caspase 3 and p53.

Total RNA was extracted using TRIzol Reagent. After homogenizing the cells with TRIzol reagent, the tubes was incubated for 10 min and centrifuged at 1000 rpm for 5 min. 200 µl of chloroform was added to the supernatant, allowed to incubate for 5min at room temperature and centrifuged at 12000 rcf for 20min. Then 500 µl of isopropyl alcohol was added to the supernatant to precipitate the total RNA and centrifuged at 12000 rcf for 15min following the incubation period of 10 min. The supernatant was decanted carefully; the pellet was washed thrice with 75% ethanol, centrifuged at 12000 rcf for 15 min.

The pellet was air dried and re-suspended in 20 µl of RNase free water and stored in -80˚C until use. RT-PCR was carried using PCR master cycler gradient (Eppendorf, Germany) and semi-quantified using Bio1D software in gel documentation (Vilber Loumart, France). Primer sequence used is shown in Table 1.

Data Analysis

Data were expressed in mean ± SEM. Mean difference between the groups were analyzed by one way ANOVA followed by turkey's multiple comparison test using graph pad prism 5.0. p value <0.05 was considered as statistically significant (*,** - indicates p< 0.05 and 0.01, respectively vs control).

Table 1: Primer sequence used in the RT-PCR

Primer	Forward Primer	Reverse Primer
GAPDH	5'-CGACAGTCAGCCGCATCTT-3'	5'-CCAATACGACCAAATCCGTTG-3'
BAX	5'- GAGTGTCTCCGGCGAATTG-3'	5'- TGGTGAGCGAGGCGGTGAG-3'
BCL2	5′- CGGGAGATCGTGATGAAGT-3′	5′- CCACCGAACTCAAAGAAGG-3′
Caspase 3	5′- AATTCAAGGGACGGGTCATG-3′	5′- GCTTGTGCGCGTACAGTTTC-3′
P53	5'- GGATGCCCGTGCTGCCGAGGAG-3'	5'- AGTGAAGGGACTAGCATTGTC-3'

RESULTS AND DISCUSSION

The present study demonstrates the anti-cancer activity of quercetin and kaempferol against A375, a human epithelial malignant melanoma cells.

Cell Proliferation Assay or MTT Assay

Cell based assays are used to determine the cytotoxic nature of the test drugs. Cell viability or cytotoxicity assay is based on the principle that incubation of test drug with viable cells results in generating a signal that gives an index on the death or viability. In the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, the viable cells with active metabolism convert MTT into a purple colored formazan product with an absorbance at 570 nm.

Death cells cannot convert substrate to product which is measured in terms of colour intensity. Though the exact mechanism of MTT reduction into formazan is not clear, but likely involves reaction with NADH or similar reducing molecules that transfer electrons to MTT[10]. Test drugs produced cell death by various mechanisms, in most cases by triggering the nulcear damage via apoptotic mechanism. In the present study, Quercetin and Kaempferol produced a concentration dependent cytotoxicity in the A375, a human epithelial malignant melanoma cells. IC50 value of Quercetin and Kaempferol was found to be 1.54 and 12.06 ng/ml, respectively, in the tested conditions (Figure 3).

From the very low IC_{50} value it can be inferred that A375, a human epithelial malignant

melanoma are highly susceptable to both Quercetin and Kaempferol.

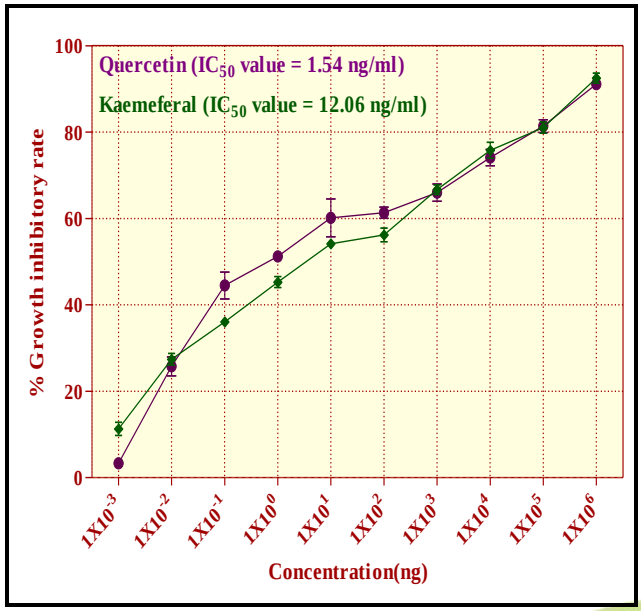

Figure 3: Effect of Quercetin and Kaempferol on growth inhibition in A375 cell lines

Quercetin and Kaempferol up-regulated bax, caspase 3 and p53 mRNA expression and also bcl2 in A375 cells

Balance between the pro- and anti-apoptotic factors determines the apoptosis programme in the cells and most chemotherapeutic interventions play a role on this balance and triggers apoptosis. Gene such as p53, caspase 3, bax and bcl2 are involved in the programmed cell death[11]. Pro-apoptotic genes such as p53, bax and caspase 3 are generally need to be up-regulated by therapeutic interventions and Bcl2, in fact, no or mild down-regulation[12]. In the preset study, semi-quantitative RT-PCR analysis revealed that the treatment with quercetin and kaempferol significantly and dose dependently up-regulated the mRNA expression of pro-apoptotic genes such as p53, bax and caspase 3 and down-regulated bcl2, a potent suppressor of apoptosis in the A375 cell lines. Further, quercetin and kaempferol increased the gene expression of caspase 3, which could be due to the stimulation on mitochondrial intrinsic pathway.

Bax is a death promotor gene in Bcl-2 family[13] which initiates apoptotic cell death via mitochondria[14]. Stimulation of mitochondrial intrinsic pathways leads to the release of cytochrome-c and activates caspase-9 in turn initiates downstream caspase cascade ultimately leading to cell death[15,16]. On the other hand, up-regulation of p53 increases the expression of Bax gene which further activates pro-caspase genes[17,18]. Caspase-3 is important to activate cytosolic endonuclease, caspase activated deoxyribonuclease that cleaves genomic DNA into oligonucleosomal fragments[19,20]. The data from the present study proved that quercetin and kaempferol are potential inhibitor of A375, human epithelial malignant melanoma cells. These compounds induced the cell death by regulating the genes involved in apoptosis. These two flavonoids may be investigated for the protective effect against radiation induced skin carcinoma in animals.

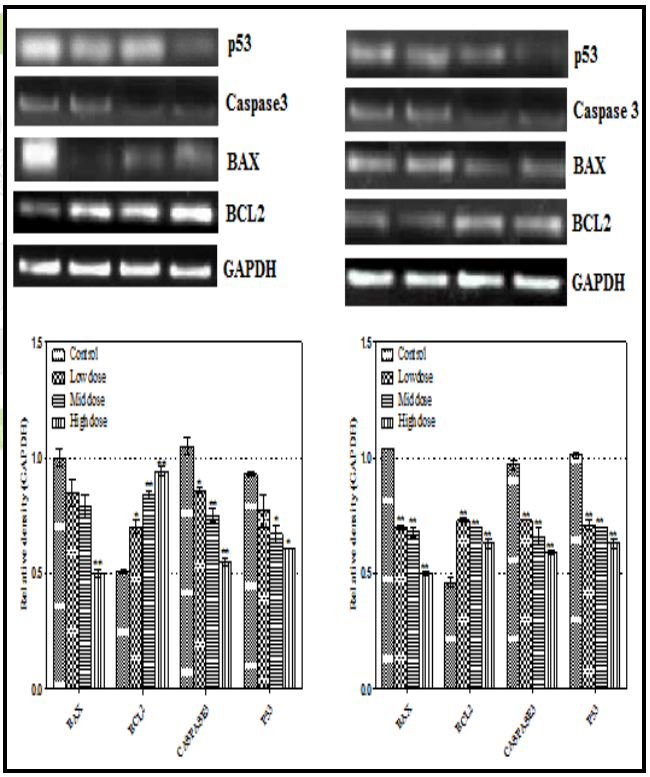

Figure 4: Effect of Quercetin and Kaempferol on apoptotic mRNA expressions in A375 cell line using MTT assay

A and C: Effect of quercetin on apoptotic mRNA expressions; lane 1 – Control; lane 2 – Low dose (0.1ng/ml); lane 3 – Mid dose (1ng/ml); lane 4 – high dose (10ng/ml). Graph representing the apoptotic expression, values were expressed in

mean ± SEM; mean difference between the groups were analyzed using one way anova followed by Tukey's multiple comparison test in graphpad prism 5.0. *, ** - indicates p< 0.05 and 0.01, respectively vs control.

B and D: Effect of Kaempferol on apoptotic mRNA expressions, respectively; lane 1 – Control; lane 2 – Low dose (1ng/ml); lane 3 – Mid dose (10ng/ml); lane 4 – high dose (100ng/ml). Graph representing the apoptotic expression, values were expressed in mean ± SEM; mean difference between the groups were analyzed using one way anova followed by Tukey's multiple comparison test in graphpad prism 5.0. *, ** - indicates p< 0.05 and 0.01, respectively vs control.

CONCLUSION

In summary, the present study demonstrated that quercetin and kaempferol has the ability to inhibit the proliferation of A375, human epithelial malignant melanoma cells. Both the compounds have the potential to up-regulate p53, Bax, Casp-3 and down regulate Bcl-2 gene and ultimately leads to cell death. Hence, these compounds may be subjected to further investigations in *in vivo* models of skin carcinoma.

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