Research Article

Flavonoids Isolated from *Foeniculum vulgare* (Fennel) have Virostatic Efficiency Against *Bluetongue Virus*

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ABSTRACT

Two flavonoids from fennel seeds quercetin and isoquercetin have been evaluated for virostatic efficacy against *Bluetongue virus* **(**BTV) in *in vitro.* They showed expressive effect on virus growth in BHK cell cultures by inhibiting the development of virus induced cytopathic effects at an effective concentration of 0.75±0.11 µM and 1.07±0.17 µM and the selective indexes was calculated accordingly and found that quercetin was highly selective than isoquercetin with SI_{50} values of 167 and 58 respectively. They exhibited low cytotoxicity on BHK cells at an inhibitory concentration of 125µM and 62.5µM respectively. These observations suggest that both the compounds are suitable for development of efficacious anti BTV agents.

Keywords: Antiviral agents for BTV, Flavonoids, Foeniculum vulgare, Quercetin and isoquercetin.

INTRODUCTION

Flavonoids are a group of polyphenolic compounds, which are widely distributed throughout the plant kingdom. Flavonoids have low toxicity in mammals and their health promoting effects in humans and animal were demonstrated earlier.[1,2] Quercetin, Iso form of quercetin and rutin are the main flavonols present in many fruits, vegetables, leafy vegetables as well as beverages. But a limited number of clinical trials have been reported on these isolated flavonols. Previous investigations proved that these compounds have health benefits attributed to their improvement of capillary health by strengthening blood vessels and acting as strong antihistamine agents.[1-6] Besides the antioxidant activity quercetin and isoquercetin are also proved to be effective virostatic agents *in vitro* against many of the human and animal viruses like HSV-1, *Poliovirus type* 1, *Influenza A* and *B*, *Parainfluenza* type 3, Adenovirus, RSV and Rhinoviruses. They exhibit virostatic efficacy by inhibiting synthesis of several lipids, enzymes like Tyrosine kinase, Serine kinase and Threonine kinases by competiting for the ATP binding sites, thus reducing the virus infection or replication.[7-13]

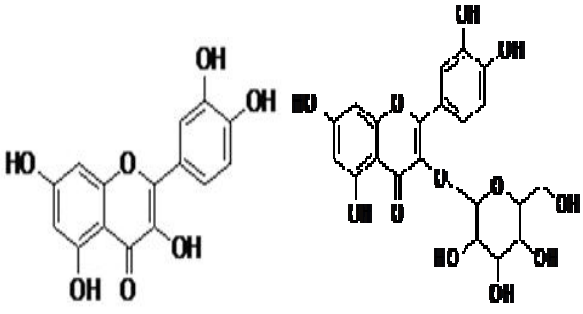


Figure 1: Chemical structure of Quercetin and Isoquercetin

Flavonoids are not only found in food and alcoholic beverages, but also in large number of commonly used medicinal plants including fennel.[1, 2] *Foeniculum vulgare* commonly known as fennel belongs to the family *Apiaceae*, is one of the widespread and a very popular medicinal and economic plants that was native to Southern Europe and Mediterranean region, but is currently cultivated in most of the temperate areas.[14] The bulb, young shoots, leaves and fully ripened and dried fruits were commonly used for homemade remedies and also in commercial herbal products. The constituent oils and extracts of fennel seed which were rich with polyphenols shown to possess considerable benefits in various areas like inflammation, asthma, indigestion, hirsutism and colic. Among the total phenolic compounds in hydroalcoholic extracts of fennel, flavonoids constitute about 12.3mg/g, rich with quercetin, isoquercetin and rutin.[14,15] In the present study, we isolated quercetin and isoquercetin from fennel grains, one of the rich source of above flavonols next to onion[2] and investigated for their antiviral activity against *Bluetongue virus* in *In vitro* using Baby Hamster Kidney Cell lines (BHK 21 cell lines) through multiple approaches.

Bluetongue disease is an infectious, non-contagious, arthropod borne viral infection of wild and domestic ruminants including sheep, goat and cattle around the world. It mainly affects sheep causing a severe systemic disorder with moderate to high mortality rate. Although it affects cattle, disease is subclinical or inapparent in cattle that act as reservoir hosts.[16] *Bluetongue virus* (BTV) is a prototype virus of genus *Orbivirus,* family *Reoviridae* and genome consists of 10 discrete segments of dsRNA surrounded by three concentric protein layers. BTV is endemic in the majority of the warmer regions around the world and are chiefly transmitted by *Culicoides* spp. BTV is causing worldwide losses that were estimated as

$3.0 billion/year, thus ranked 'A' by Office international des Epizooties (OIE). Due to its economic importance, BTV has become the subject of extensive molecular, genetic and structural studies and now represents one of the well characterized viruses.[17, 18]

An astonishing fact is that, there are no suitable antiviral drugs and effective control measures developed against BTV infection till today. At present, vector control, animal quarantine and vaccination using BTV virus like particles are the main preventive strategies available to prevent the possible BTV epidemics.[17,18] However, due to change in vector habitats and development of new virus strains, BTV is still endemic in many regions of the world. Even though vaccination is most effective preventive measure during outbreak, protection of an animal from the threat may not occur more than two weeks of initial vaccination.[17] Hence, there is a pressing need to develop an antiviral drug which can be offered as a therapeutic agent during outbreaks. Recent studies have discovered potential anti BTV drugs and further implementation is under progress.[17,18] They identified 185 compound structures grouped into six analog series corresponding to six scaffolds enriched within the active set compared to their distribution in the NIH molecular library. In this context, we selected active compound groups in particular to polyphenols which were easily available in nature to study the virostatic efficacy against BTV.

MATERIALS AND METHODS

Isolation of quercetin and isoquercetin from Foeniculum vulgare (Fennel)

Air dried fennel grains were ground to fine powder and mixed with 70% ethanol solution using cold maceration technique. The extract was filtered, and the filtrate was evaporated by rotary vacuum evaporator. The percentage yield of the ethanol extract was found to (20.22% w/w).[24-35] The dried ethanol extract was suspended in water, mixed with n-hexane in a separating funnel and the n-hexane portion was discarded after separation. To the aqueous portion, dichloromethane was added in separating funnel, after separation dichloromethane portion was discarded. To the aqueous portion ethyl acetate was added and ethyl acetate portion was collected. Then solvent was completely removed by rotary vacuum evaporator. The yield of the ethyl acetate fraction was estimated 2.45% w/w. Finally ethyl acetate fraction was subjected to qualitative chemical test and thin layer chromatography studies and showed positive test for flavonoids. The structure of the quercetin and isoquercetin were determined on the basis of 1H NMR and 13C NMR.

(3,5,7-trihydroxy-2-(3,4-dihydroxyphenyl)-4H-chromen-4-one)

Yellow solid, m.p. = 324–326 ºC; ^{1}H NMR (DMSO–d_6, 400 MHz) δ 6.19 (s, 1H, H–9), 6.41 (s, 1H, H–7), 6.89 (d, 1H, H–15, J=8.0 Hz), 7.54 (dd, 1H, H–16, J=8.0 Hz), 7.68 (d, 1H, H–12, J=2.0 Hz), 9.32 (br s, 1H, OH–22), 9.34 (br s, 1H, OH–21), 9.38 (br s, 1H, OH–19), 10.74 (br s, 1H, OH–20), 12.48 (br s, 1H, OH–17) ^{13}C NMR (DMSO–d_6,100 MHz): δ 93.33 (C–9), 98.17 (C–7), 103.00 (C–5), 115.08 (C–12), 115.58 (C–15), 119.96 (C–16), 121.96 (C–11), 135.68 (C–3), 145.03 (C–14), 146.81 (C–13), 147.68 (C–2), 156.14 (C–6), 160.70 (C–10), 163.83 (C–8) and 175.81 (C–4); LC–MS (positive ion mode): m/z 303(M+H)$^+$ for $C_{15}H_{10}O_7$ (Fig. 1(a)).

(5,7-dihydroxy-2-(3,4-dihydroxyphenyl)-3-(tetrahydro-3,4,5-trihydroxy-6-(hydroxymethyl)-2H-pyran-2-yloxy)-4H-chromen-4-one)

Pale yellow solid, ^{1}H NMR (DMSO–d_6, 400 MHz) δ 3.18 (m, 2H, H-3'' and H-4''), 3.40 (m, 1H, H-5''), 3.42 (m, 1H, H-2''), 3.75 (d, 2H, J=11.0 Hz, H-6''), 5.68 (d, 1H, J=8.0 Hz, H-1''), 6.37 (d, 1H, J=2.0 Hz, H-8), 6.54 (d, 1H, J=2.0 Hz, H-6), 7.06 (d, 1H, J=8.0 Hz, H-5'), 7.92 (d, 1H, J=2.0 Hz, H-2'), 8.26 (dd, 1H, J=8.0 Hz, H-6'), 9.13 (br s, 4H, 4-OH) and 12.96 (br s, 4H, 4-OH); ^{13}C NMR (DMSO–d_6,100 MHz): δ 61.02 (C-6''), 70.04 (C-4''), 74.25 (C-2''), 76.63 (C-3''), 77.57 (C-5''), 93.72 (C-8), 99.06 (C-6), 101.10 (C-1''), 115.39 (C-5'), 116.21 (C-2'), 121.28 (C-1'), 121.76 (C-6'), 133.44 (C-3), 144.92 (C-3'), 148.63 (C-4'), 156.24 (C-2), 156.52 (C-9), 161.38 (C-5), 165.0 (C- 7) and 177.46 (C-4); LC–MS (positive ion mode): m/z 465 (M+H)$^+$ for $C_{21}H_{20}O_{12}$ (Fig 1(b)).

The above physical and chemical characteristics were compared with previous reported data [9].

Cells and virus stock

BHK 21 cell lines were obtained from Molecular Virology Laboratory, FMD Research centre, IVRI, Bangalore. The cells were grown in Dulbeco's Modified Eagles Medium (DMEM) supplemented with 10% heat inactivated foetal bovine serum (Hi-Media Co., Mumbai, India) and antibiotics Penicillin (100 IU/mL), Streptomycin (100 µg/mL). BTV-9 strain K8 was obtained from Department of Microbiology, College of Veterinary Science, Rajendranagar, Hyderabad. Virus was propagated in BHK21 cell lines and used at a concentration of 2.6x10^5 $TCID_{50}$ in all *In vitro* experiments.

Cytotoxicity assay

Two compounds were dissolved in DMSO and made up to a final concentration of 10mg/10ml followed by filtration through 0.2µm filters (Sartorius). Each compound was serially diluted (two fold dilution) to evaluate cytotoxicity induced by compounds.

Confluent monolayer of BHK 21 cell lines were prepared as cell suspension by trypsinization and seeded at a concentration of 5000 cells/well in a 96 well tissue culture plate. Plates were incubated at 37°C in a CO_2 incubator for 24-48hrs. After observing the monolayer, growth medium was removed and cells were washed twice with FCS free MEM. Quadruplicate wells of confluent monolayers of BHK 21 cells were incubated with different concentration of the test compounds up to 72hrs and cell viability was examined by ability of the cells to cleave the tetrazolium

salt MTT [3-(4,5-dimethyl thiazol-2ol)-2,5 diphenyltetrazoliumbromide), Sigma-Aldrich, USA], by the mitochondrial succinate dehydrogenase which develops a formazan blue colour product. Intensity of colour was directly proportional to the concentration of test compound. The 50% inhibitory concentration (IC_{50}) was calculated by regression analysis at which IC_{50} of the test compound was minimum. It was further checked by plating efficiency of the cells with the subtoxic dose of that test compound. [36-40, 4]

Screening the compounds for antiviral activity

BHK 21 cells seeded at a concentration of 5000 cells/well in 96 well tissue culture plates along with different concentrations of test compounds and BTV ($2.6x10^5$ $TCID_{50}$). Cells without compound and cells with BTV were used as cell control and virus control respectively. Each assay was tested in triplicates. Plates were incubated at 37ºC at 5% CO_2 atmosphere and observed up to 72hrs. Specific cytopathic changes induced by BTV were observed at a time intervals of 24, 48 and 72hour of post inoculation [36-40,4]. The 50% effective antiviral concentration (EC_{50}) was evaluated through neutral red dye method. Optical densities were measured at 540nm which directly related to percentage of viable cells.[41]

Amplification of test compound treated BTV infected cell lines by RT-PCR

BHK21 cells in $25cm^2$ tissue culture flasks along with the different concentrations of test compounds and BTV also maintained to evaluate the virus progress in cell cultures by molecular approach (as there was no commercially available antiviral agents to maintain positive control). Culture supernatant was removed from the flasks at a time intervals of 24, 48 and 72 hrs of post inoculation (PI). Total RNA was isolated by using Trizol reagent (Sigma Aldrich). Presence of BTV RNA in cell cultures which were inoculated along with test compounds were confirmed by RT-PCR method by using NS3 specific primers (NSP3FP-5'-GCGGGATCCATGCTATCCGGGCTGAT-3'; NSP3RP GCGTACGATGCGAATGCAGC-3').[36-39]

RESULTS

The present study was carried out to test the virostatic efficacy of fennel extracted quercetin and isoquercetin compounds against *Bluetongue virus*. Antiviral activity of these two compounds against BTV was assessed by calculating the 50% effective concentration (EC_{50}) and Selective indexes (SI_{50}). Before evaluating the antiviral activity, the effective dose at which toxicity of compounds 50% (IC_{50}) for cell growth were calculated as 125µM for quercetin and 62.5µM for isoquercetin by plotting a graph against compound concentration and cell viability respectively. When cells were subcultured along with effective concentration of both the compounds for three passages, no effect on cell viability and growth were noticed. These changes were compared with cell control having cells+ 0.1% DMSO.

Various concentrations of both the compounds were made to determine at which concentration they inhibit the virus induced cytopathic changes effectively against at a fixed virus concentration of $2.6x10^5$ $TCID_{50}$. When BTV infects mammalian cells (including Vero, HeLa, BHK or BSR cells) it induces apoptosis and variety of cytopathic changes characterized by cell shrinkage, membrane blebbing and DNA fragmentation.[18] Quercetin and Isoquercetin showed significant virostatic efficacy by inhibiting cytopathic changes induced by BTV in cell lines when compared to virus control.

In the absence of commercially available suitable virostatic agent (to maintain positive control), confirmation was done by performing the NS3 RT-PCR through isolation of viral genome from cell culture fluid at different time intervals (0, 24, 48 and 72 hrs of PI) using BTV-NS3 specific primers. Drug treated, BTV infected cultures failed to show the PCR amplification after 24hrs post inoculation (Figure 3a & 3b), whereas BTV infected positive control showed 250bp amplified product confirming the reduction in virus growth and replication (Figure 2, 3a & 3b). Using the ten-concentration dose response assay (Log_{10}), the EC_{50} of quercetin and isoquercetin were determined at 0.75±0.11µM and 1.07±0.17µM respectively (Fig.4). Selective indexes (SI_{50}) were calculated accordingly which equals to EC_{50}/IC_{50}values as 167 for quercetin and 58 for isoquercetin respectively (Table 1), indicating that both the compounds were selective against BTV. According to the SI_{50} values it was also observed that quercetin was highly selective than isoquercetin.

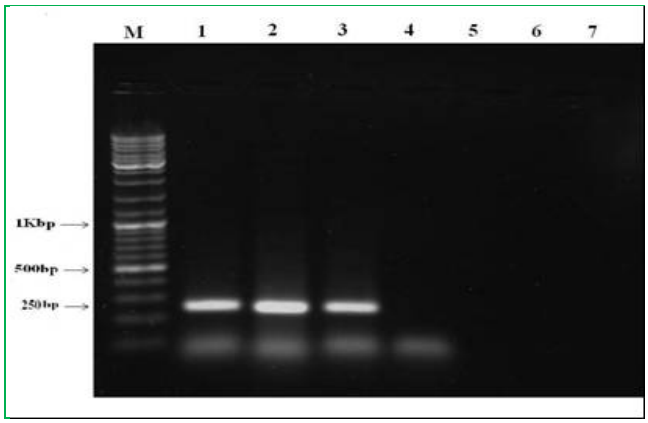
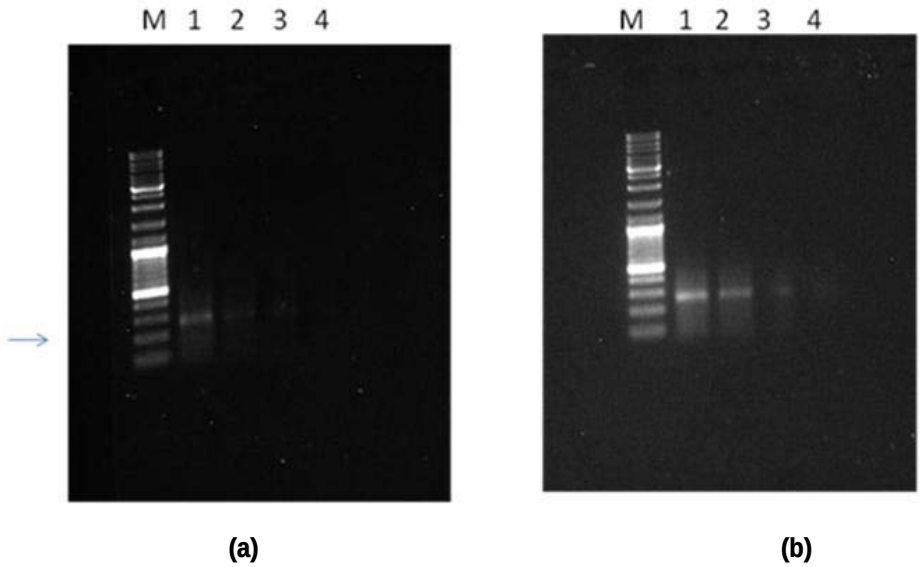


Figure 2: Detection of BTV in virus controls by NS3 RT-PCR amplification

Lane M: Double digested DNA ladder; Lane 1, 2 & 3: Cells + Virus (Virus control) 250 bp of NS3 RT-PCR; Amplified product at different time Intervals (24h, 48h and 72h); Lane 4: Negative Control

DISCUSSION

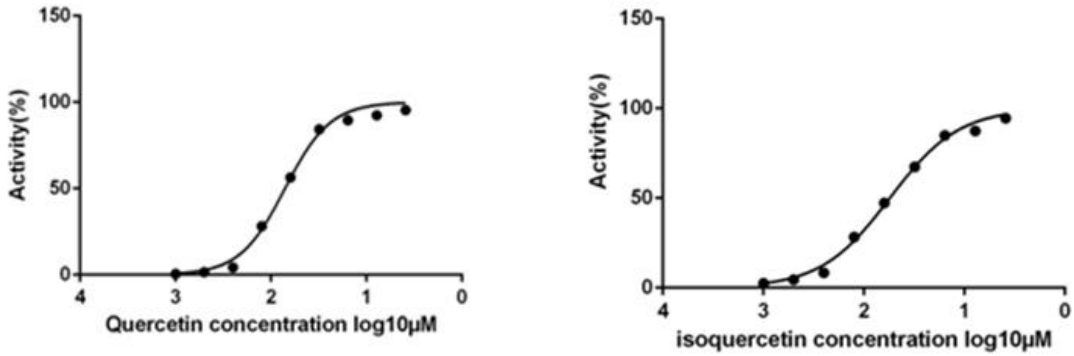
In addition to the antioxidant activity, the antiviral activities of Quercetin and Isoquercetin against a number of human/animal viruses have been studied in time and again at both *In vitro* and *In vivo* level. The role of several naturally occurring dietary flavonoids (including quercetin) on the infectivity and replication of herpes



(a) (b)

Figure 3: RT-PCR confirmation of Virus progress in drug treated cell lines

Figure 3: a) Cells +Quercetin (125µg) + BTV At different time intervals Lane M- 1 Kb DNA Ladders Lane 1 to 4 – 0hr, 24hr, 48hr and 72hr
Figure 3: b) Cells + Isoquercetin (62.5 µg) + BTV at different time intervals Lane M: 1 Kb DNA ladder Lane 1 to 4: 0hr, 24hr, 48hr and 72hr.



(a) (b)

Figure 4: Anti BTV activity of fennel extracted Quercetin and Isoquercetin were tested at various concentrations in BHK-21 cell lines after 72hrs PI . Each data point represents mean and standard deviation from three individual experiments.

simplex virus type 1, polio-virus type 1, parainfluenza virus type 3 and respiratory syncytial virus have been demonstrated using plaque reduction assays.[11] Naturally occurring compounds of 4'-Hydroxy-3-methoxyflavones and their synthesized analogs were evaluated for virucidal activity against poliovirus (type 1 and 11) and rhinovirus and also proved that quercetin was not mutagenic in concentrations up to 2.5mg/ml.[19] It was also reported that antiviral effect of quercetin may be mediated through induction of interferon. A recent study revealed that quercetin induces the gene expression and production of helper T lymphocyte-1 (Th-1)-derived IFNγ, and it down regulates Th-2-derived IL-4 when cultured with human peripheral blood mononuclear cells.[20] Quercetin was isolated from *Caesalpinia pulcherrima* (a traditional Chinese medicinal plant) and its antiviral activity has been proved against human herpesviruses 1 and 2 and adenoviruses (serotypes 3,8 and 11) through the inhibition of their multiplication in vitro.[10] Through multiple approaches such as molecular docking, SPR, FRET bioassays and mutagenic studies it was reported that quercetin-3-β-galactosidase and eight quercetin analogs bind to SARS-CoV 3C-like protease enzyme which involves in proteolytic release of replicative proteins from precursor polyproteins and inhibit the SARS virus replication.[21] Studies using animal models revealed that short term feeding of quercetin before influenza infection in mice can delay the onset of sickness and reduced the inflammation in upper respiratory tract.[22] Morbidity, symptoms severity and mortality rates were lowered in quercetin treated mice when compared to the control. *In vitro* evaluation of quercetin in treatment of HCV lowered the intracellular viral accumulation, protein translation and release of progeny viruses.[23] Studies documenting the reduced influenza infection in mice using isoquercetin were also observed.[8] With respect to results obtained in

the present study, there was also evident that these two compounds effectively suppressed the bluetongue virus *In vitro*. Hence, they can recommended for develop as suitable anti BTV drugs. However, it yet to be determines the mechanical action of these two compounds on virus and host systems by using suitable animal models.

Table 1: Assessment of cytotoxicity and anti BTV activity of fennel extracted flavonoids in BHK21 cells

Compound	IC_{50} (µM)	C_{50}(µM)	Selective Index(SI_{50})
Quercetin	125	0.75±0.11	167
Isoquercetin	62.5	1.07±0.17	58

CONCLUSION

In conclusion this is only a preliminary study reporting antiviral activity of quercetin and isoquercetin isolated from *Foeniculum vulgare (Fennel)* against BTV. Quercetin and isoquercetin may cater the need of antiviral agent at the time of outbreaks where the vaccination fails to reduce the possible epidemics. However, further studies are required to know the mechanistic action of these compounds using suitable animal models.

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