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Review

Effect of the Plant Matrix on the Uptake of Luteolin Derivatives-containing *Artemisia afra* Aqueous-extract in Caco-2 cells

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ABSTRACT

Aim of the study: Luteolin is a major flavonoid constituent and a primary candidate that might contribute to the claimed *in vivo* protective effects of *Artemisia afra* (Jacq. Ex. Willd). However, an exhaustive search yielded no literature evidence on the absorption, metabolism and fate of this flavonoid from the traditional plant preparation. The purpose of this study was to investigate the effect of the plant matrix on the uptake of luteolin derivatives from *Artemisia afra* aqueous extract in human intestinal epithelial Caco-2 cells.

Materials and methods: Cell monolayers were incubated with 5, 10 and 20 μg/ml doses of luteolin aglycone, luteolin-7-0-glucoside, un-hydrolyzed or acid-hydrolyzed *Artemisia afra* extracts, and samples of 150 μl each were collected from both apical and basolateral sides of cells at 30, 60 and 120 min for HPLC and LC–MS analyses.

Results: After 1-h exposure, the uptake of luteolin aglycone and luteolin-7-0-glucoside from the un-hydrolyzed and acid-hydrolyzed extracts was significantly faster and quantitatively higher (i.e. >77% vs. <25% of the initial doses over the first 30 min, $p < 0.05$) than that from non-plant solutions. Apical to basolateral permeability coefficients for luteolin and its-7-0-glucoside in the extracts were 1.6- to 2-fold higher than that for the non-plant solutions. Glucuronidation was an important pathway of metabolism for luteolin in both non-plant and plant extract forms.

Conclusions: Luteolin in *Artemisia afra* aqueous extract, regardless of its form (i.e. whether aglycone and 7-0-glucoside), is taken up better and more efficiently metabolized than the aglycone and 7-0-glucoside forms administered as pure solutions in Caco-2 cells. Flavonoid actives from *Artemisia afra* plant extracts and especially traditionally prepared dosage forms may thus have better bioavailability, and consequently greater *in vivo* potency, than that predicted from studies done using the pure solutions.

Contents

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1. Introduction

Artemisia afra is a well-known medicinal plant of South Africa, where it is known as "Wilde als" (Roberts, 1990). It is widely used for numerous ailments including colds, coughs, diabetes mellitus, heartburns, bronchitis and asthma among others (Van Wyk et al., 2000; Watt and Breyer-Brandwijk, 1962). However, very little is known about the active chemical constituents responsible for the pharmacological activities associated with the plant. Chemical analyses conducted on extracts of *Artemisia afra* have indicated the plant to contain acetylenes, coumarins, flavonoids (i.e. apigenin, luteolin, quercetin, etc.), terpenoids and volatile oil (Van Wyk et al., 2000; Watt and Breyer-Brandwijk, 1962; Waithaka, 2004). Of these, flavonoids most likely have the best potential to contribute to the major activities of *Artemisia afra*.

Flavonoids are polyphenolic compounds that occur naturally in several plants as secondary metabolites (Pong et al., 2005). Epidemiological studies have suggested that flavonoids have many health protecting effects, including anticancer and anti-viral (Pong et al., 2005), anti-osteoporotic and anti-cardiovascular activities (Murota et al., 2002). Other potentially beneficial activities are anti-inflammatory, anti-allergic (Silberberg et al., 2006), anti-microbial, anti-proliferative, chemo-protective and antioxidant effects (Benavente-Garcia et al., 1997). In fact, it is generally assumed that flavonoids (antioxidant nutrients) are the primary candidates contributing to the claimed protective effects associated with plant medicines. However, for flavonoids to be the *in vivo* active constituents of plants such as *Artemisia afra*, they must be bioavailable i.e. be effectively absorbed into the blood stream.

Few studies have addressed the ability of flavonoids to reach proposed *in vivo* sites of action. The limited number of studies conducted in humans and rats has indicated very poor and variable intestinal absorption of phenolic compounds (Walgren et al., 1998). Flavonoids occur in plants either in the form of aglycones or glycosides. Pure flavonoid aglycones have been reported as being absorbed freely from the gut by passive diffusion, while the flavonoid glycosides must usually be hydrolyzed (by intestinal enzymes and/or by colonic microflora) to the corresponding aglycone molecules prior to their gastrointestinal absorption (Manach et al., 2004; Pong et al., 2005). However, phloretin and quercetin glycosides have, after being administered as such, also recently been shown to occur as glycosides in human plasma, and the glycoside genistin was partially absorbed without previous cleavage (Andlauer et al., 2000).

Currently, the situation is even more confusing when it comes to the absorption of flavonoids from natural products (i.e. those in plant or fruit extracts), specifically whether natural flavonoid glycosides can also be directly absorbed from the gastrointestinal tract or whether they are hydrolyzed in the small intestine prior to absorption (Spencer et al., 1999). A number of early studies hypothesized that flavonoids would not enter the circulation, either as the natural glycosides or as the aglycone hydrolysis products. It was thought that cleavage at the central heterocyclic ring by intestinal bacteria would occur thereby effectively destroying the antioxidant properties of the compounds and generating phenolic acid fission products (Spencer et al., 1999). However, in a recent study the glycoside form of quercetin (rutin) was detected in the circulation after the consumption of apple or onion, and in blood of individuals consuming a generally high fruit and vegetable diet (Spencer et al., 1999). Anthocyanidin glycoside (anthocyanin) absorption has also been reported in human plasma and the direct intestinal absorption of cyanidin-3-glycoside derived from red fruit, elderberries and blackcurrants, has been detected in the plasma of both rats and man (Spencer et al., 1999; Bitsch et al., 2004). On the other hand, luteolin-7-0-glycoside, although present in Artichoke, was not found in human plasma or urine after the administration of the leaf extracts. Also, no free luteolin could be detected in plasma or urine prior to enzymatic hydrolysis of conjugates. However, after Beta-glucuronidase treatment, luteolin was identified in the human plasma (Wittemer et al., 2005). This and other observations in the Caco-2 cell model, suggest that glucuronidation or sulfation could be major metabolic pathways for flavonoids in the gut wall (Walgren et al., 1998; Wittemer et al., 2005). Taken together the issue whether natural flavonoids are absorbed as aglycones and/or glycosides are still unresolved and probably also complicated by simultaneous metabolism (i.e. glucuronidation and/or sulfation) that may occur during absorption (i.e. the passage from the gut into the circulation).

The mechanism via which the flavonoids, whether as aglycone or glycosides, can be taken up into the cells of the gastrointestinal tract (GIT) has also not yet been fully resolved. It is generally assumed that the aglycones of the flavonoids are absorbed mainly by passive diffusion (Pong et al., 2005). However, from studies conducted *in vitro* and in humans it was concluded that the absorption of some flavonoids (e.g. quercetin) was enhanced by conjugation with glucose and possibly by absorption via the glucose carrier (glucose transporter: SGLT1) (Andlauer et al., 2000; Liu and Hu, 2002). Also other compounds, such as flavonols, may enter the cell as intact glycosides via the sodium-dependent glucose transporter (SGTL1) (Manach et al., 2004; Wittemer et al., 2005). The mechanism of uptake of the various forms of flavonoids thus needs further investigation.

Besides the above-mentioned issues, the effect the plant material matrix can have on the absorption or on transit (i.e. GIT) metabolism and, consequently, the bioavailability of flavonoids, has also not yet been fully investigated (Manach et al., 2004; Wittemer et al., 2005). Direct interactions between flavonoids and some components within the plant matrix, such as binding to polysaccharides and other chemical constituents, have been reported for

some medicinal plants (Manach et al., 2004; Pong et al., 2005), and these interactions are said to affect the absorption of phenolic compounds (Manach et al., 2004; Pong et al., 2005; Wittemer et al., 2005). Moreover, the plant matrix can affect various parameters of gut physiology (e.g. pH, intestinal fermentations, biliary excretion, transit time, biological fluids, etc.) and the intestinal microflora which in turn may have effects on the absorption of flavonoids. The carriers and enzymes involved in flavonoid absorption and metabolism may also be induced or inhibited by the presence of some micronutrients or xenobiotics within the plant matrix (Manach et al., 2004; Wittemer et al., 2005). Overall, the effect that plant matrices may have on the uptake and bioavailability of flavonoids may thus be quite complex.

Recently, we have found that the bioavailability of luteolin from aqueous extract of *Artemisia afra* in the vervet monkey was significantly better than in its pure form (aglycone) (Muganga, 2004). This may be due to the form of luteolin in the plant material or a quick conversion of the glycoside luteolin to its aglycone form prior to absorption.

This investigation focussed on the effect the structure (i.e. whether aglycone or glycoside) of the flavonoid and the plant matrix may have on the bioavailability of flavonoids from a traditional plant medicine. Specifically, the intestinal disposition of the flavone luteolin in aglycone, in glucoside and in *Artemisia afra* plant extract forms was investigated in human intestinal epithelial Caco-2 cells. It was anticipated that the luteolin in *Artemisia afra* aqueous extract, regardless of its form, would have a better uptake in the Caco-2 cells than the luteolin in the absence of the plant matrix.

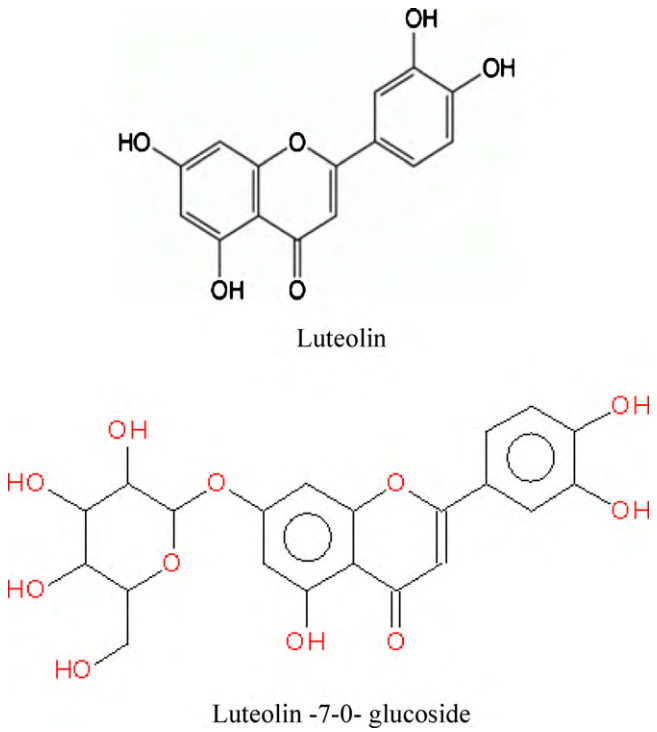

Fig. 1. Chemical structures of luteolin aglycone and its 7-glucoside.

2. Materials and methods

2.1. Materials

Caco-2 cells were purchased from Highveld Biological Association (Johannesburg, South Africa). Luteolin aglycone and luteolin-7-0-glucoside (Fig. 1) were purchased from Sigma (St. Louis, USA) and Extrasynthese (Genay, France), respectively. Millicell®-ERS (Electrical Resistance System) and Millicell-PCF inserts (polycarbonate membrane, 0.4 μm pore-size, 12 mm I.D.) were from Millipore Corporation (Bedford, MA, USA). MITO+™ Serum Extender was supplied by Becton Dickinson Bioscience (Bedford, MA). Dulbecco's modified Eagle's Medium with 4500 mg/l glucose and L-glutamine (DMEM), Dulbecco's phosphate buffered saline without calcium or magnesium (DPBS), foetal calf serum (FCS), nonessential amino acids (NEAA), penicillin–streptomycin and trypsin (0.25%) were obtained from Bio Whittaker® (Lonza, Walkersville, USA). All other materials and reagents were either of analytical grade or of the highest purity and used as received.

2.2. Preparation of the aqueous extract

Freshly picked *Artemisia afra* plant material was purchased from Montague garden (Western Cape Province, South Africa). The material was identified as *Artemisia afra* by Mr. Franz Weitz, botanist in the Department of Botany at the University of the Western Cape (UWC) and a voucher specimen (number 6640) was dried and deposited in the herbarium at UWC. Thereafter, fresh leaves of *Artemisia afra* plucked form the stalks were rinsed with distilled water, dried in the oven at 30 °C and slightly crushed (but not powered) by hand. The aqueous extract was prepared in a way that, as much as possible, mimicked the method traditional herbal practitioners use to extract their plant medicines. The dried leaves were suspended in distilled water (50 g dried leaves per liter of water) and the mixture boiled for 30 min (Mukinda and Syce, 2007). The decoction obtained was cooled, filtered, frozen at −80 °C and then freeze-dried. The yield of the crude aqueous extract was about 24% (w/w). The final *Artemisia afra* extract collected was sterilized by gamma irradiation (Hepro Cape Gamma, Cape Town) and stored at −20 °C until further bioassay. In addition to the crude aqueous extract (un-hydrolyzed extract), the acid-hydrolyzed extract was also prepared as a glycoside free extract. 4 g of the crude aqueous extract was suspended in 5 ml methanol, vortex-mixed for 2 min, 8 ml of 2N hydrochloric acid (HCl) added and the mixture acid–methanol–plant heated in water bath at 80 °C for 40 min. To cooled hydrolyzed material, a solution of 1N sodium hydroxide (NaOH) was added to adjust the pH at 7, the mixture concentrated using rotor-evaporator and the final hydrolyzed extract stored at −20 °C for further biological experiment.

2.3. Preparation of standard solutions

Separate stock solutions of both luteolin aglycone and luteolin-7-0-glucoside were prepared beforehand by dissolving accurate amount (3 mg/ml) of each in dimethyl sulfoxide (DMSO). All solutions were vortexed, sonicated and the stored at −20 °C until further use. Working solutions were later prepared by dilution of stock solutions with either mobile or medium to provide a series of analytical standards ranging from 1 to 20 μg/ml for use in building of calibration curves for each authentic analyte.

2.4. Stability of the selected flavonoids and Artemisia afra aqueous extract in cell culture medium

A single concentration (5 μg/ml) of each treatment (i.e. luteolin aglycone, luteolin-7-0-glucoside, un-hydrolyzed and acid-hydrolyzed extracts) was prepared in the medium (differentiation medium (DM) consisting of serum-free DMEM with MITO+™ serum extender) at pH 7.2 and kept at 37 °C in water bath. Flavonoids were prepared by dilution of their DMSO stock solutions and plant extract by directly dissolution in the medium (in terms of its equivalent concentration of luteolin aglycone). Samples

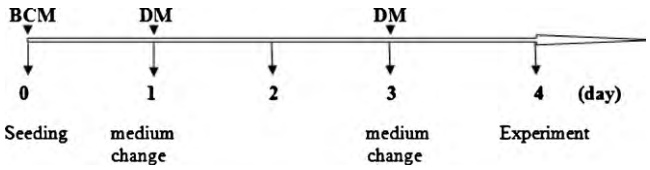

Fig. 2. Protocol for short-term Caco-2 cell culture. MITO+ was used at 1 ml per liter of serum-free DMEM.

were then collected at pre-determined time points (0, 30, 60, 120 and 240 min). All samples were in triplicate and the concentration of each compound in DM was determined by HPLC (as described in Section 2.7). The stability profile was obtained by plotting the percentage of unchanged compound vs. time.

2.5. Caco-2 cell culture

Caco-2 cells were grown in flat bottom culture flasks (25 cm^2; 50 ml) at 37 °C in a humidified atmosphere of a 5% (v/v) CO_2 in air using Dulbecco's modified Eagle's medium (DMEM) at pH 7.2. The medium was supplemented with 10% foetal calf serum, 1% nonessential amino acids, 1% sodium pyruvate and 2% penicillin–streptomycin (basic culture medium, BCM). For the multiplication of cells, the medium was initially replaced every 24 h after reconstitution of cells and thereafter every 48 h to boost the growth until the flasks reached 90% confluence. The monolayers were washed with Dulbecco's phosphate buffered saline without calcium or magnesium (DPBS) and removed from the flasks after incubation with 0.25% trypsin in 1 mM EDTA solution for 3–5 min at 37 °C. Cells were thereafter collected, centrifuged for 3 min at 2000 rpm and the residual pellet resuspended in new aliquots of DMEM for subculture purposes.

2.5.1. Short-term Caco-2 cell culture

Caco-2 cell monolayers, which were utilized in the uptake study, were obtained from a short-term cell culture experiment (4 days). Cells were seeded on isopore polycarbonate membrane of Millicell-PCF inserts (12 mm; 0.4 μm pores; growth area: 0.6 cm^2; 24 wells/plate) at a density of 1×10^5 cells/insert. In addition to the basic culture medium (BCM), differentiation medium (DM) consisting of serum-free DMEM with MITO+™ serum extender was used according to the culture protocol in Fig. 2 (Yamashita et al., 2002). (DMEM with MITO+™ Serum extender, is lyophilized from a solution of Dulbecco's phosphate buffered saline containing epidermal growth factor, transferrin, insulin, endothelial cell growth supplement, triiodothyronine, hydrocortisone, progesterone, testosterone, estradiol-17, selenious acid, and O-phosphorylethanolamine (product specification sheet, 2009, Becton Dickinson, Bedford, MA).)

The Caco-2 cells cultured in the basic culture medium (BCM) when microscopically compared to the counterpart in the differentiation medium (DM), it was observed that cells growing in the DM were morphologically bigger in size than their counterparts in the BCM.

2.5.2. Effect of luteolin and Artemisia afra aqueous extract on Caco-2 cells viability

After 25 days in culture, prior to uptake experiments, Caco-2 monolayers were firstly used in the cytotoxicity screening of luteolin aglycone and *Artemisia afra* aqueous extract to test the effect of these latter solutions on cell viability. Cells were seeded in 24-well tissue culture plates at a seeding density of 1×10^5 cells/ml and allowed to attach for 24 h. Thereafter, they were subjected to single doses of 5, 10, 20 and 30 μg/ml of either luteolin aglycone or its equivalent in aqueous extract form freshly prepared and aseptically suspended in basic culture medium (BCM). The experiment was performed in triplicate and all cells were treated over a period of 0–150 min (camptothecin, 2 μM was used as positive control). After the incubation period, all cells were washed with DPBS and then resuspended in binding buffer (in a 15 ml tube) to which 200 μl of annexin-V PE dye was added. The mixture was vortexed and incubated for 30 min. After 30 min staining, cells were washed with a fresh buffer (1 ml) to remove un-trapped dye, resuspended again in the buffer (200 μl) and analyzed by flow cytometry using 488 nm wavelength. The fluorescence and physical properties of Caco-2 cells were analyzed by the analog–digital converter (ADC) and processed using integrated software (Becton Dickinson Bioscience FACS calibur™ software, San Jose, CA, USA). Cell viability is shown graphically as percentage of the control value.

2.5.3. Caco-2 cell monolayer integrity

Measurement of transepithelial electrical resistance (TEER) was used to monitor the integrity of the cell monolayers. The resistance of cell monolayers was routinely recorded before and after the experiment using a Millicell®-ERS system. To calculate the TEER of the cell monolayer, the electrical resistances of the polycarbonate membrane of insert and medium measured before the seeding were subtracted from the total electrical resistances determined with the monolayer. Only Caco-2 cell monolayers having TEER values exceeding 180 Ωcm^2 were utilized in the uptake study.

2.6. Uptake and metabolism study of luteolin aglycone, luteolin-7-0-glucoside and Artemisia afra un-hydrolyzed and acid-hydrolyzed aqueous extracts in Caco-2 cells

Loading solutions (5, 10 and 20 μg/ml) of either luteolin aglycone or luteolin-7-0-glucoside or equivalent luteolin aglycone in *Artemisia afra* un-hydrolyzed and acid-hydrolyzed aqueous extract forms were prepared by dilution of accurate volumes of their stock solutions in DM. The prepared solutions were aseptically filtered through a 0.45 μm sterile syringe filter. Following the removal of the culture medium and rinsing with DPBS, 500 μl of each loading solution for each selected treatment was added to the donor side (apical of the Millicell-PCF insert), 500 μl of the blank medium (DM) injected to the receiver side (basolateral side or in the well) and the plates were then incubated at 37 °C in 5% CO_2 and 95% humidified air. Samples of 150 μl each were taken from both apical and basolateral sides at different time intervals of 30, 60 and 120 min. The experiment was performed in triplicate. HPLC and LC–MS were used to determine the levels of unchanged compounds and the metabolite profiles form both apical and basolateral sides of cells and thereafter the apparent permeability coefficient (P_{app}) and percentage of recovery calculated (% of initial dose of treatment).

2.7. Samples preparation

2.7.1. Plant sample

To determine luteolin aglycone and its glycoside contents in the plant extract the following procedure was used. Briefly, 25 mg of the crude plant extract was suspended in 200 μl methanol and vortex-mixed for 1 min (un-hydrolyzed sample). To obtain the acid-hydrolyzed plant extract, 4 ml of 2N hydrochloric acid (HCl) was added, and the mixture acid–methanol–plant heated in water bath at 80 °C for 40 min. To un-hydrolyzed and cooled acid-hydrolyzed samples, 5 ml ethyl acetate was added to extract the actives (flavonoids), the mixtures vortex-mixed for 3 min and then centrifuged for 10 min at 3000 rpm. The supernatant was collected, evaporated to dryness under a gentle stream of nitrogen gas, the residue reconstituted in 200 μl of HPLC mobile phase and 20 μl injected onto the HPLC column. The flavonoids levels (i.e. luteolin

aglycone and luteolin-7-0-glucoside) were calculated using standard curves for each analyte.

2.7.2. Samples from transport studies

During the uptake experiment, samples were collected into 2 ml sterile tubes and immediately put on ice. At the end of the experiment, all samples were centrifuged for 5 min at 2000 rpm, supernatants collected and stored at −20 °C. To determine the levels of luteolin aglycone, luteolin glucoside and the metabolite profiles in the collected samples, the frozen samples were thawed and 40 μl injected onto the HPLC–LC–MS column.

2.8. Analytical methods

Prior to the transepithelial uptake experiment study, the *Artemisia afra* aqueous extract was tested for the presence and levels of both luteolin aglycone and its glycosides. After the experiment, the same manner, all the samples were tested for levels of parent compounds and profile of metabolites using HPLC and LC–MS systems.

The HPLC conditions were as follows: System, Agilent 1200 controlled by chemstation software with a DAD detector and an autosampler (Agilent Technologies, CA); column, synergy®, hydro-reverse phase C18 column (250 mm × 4.60 mm, Phenomenex 80A, USA); mobile phase consisted of acetonitrile and 0.2% formic acid (FA); injection volume, 20 or 40 μl; flow rate, 1 ml/min; column *T* (°C), 20 °C; DAD detector wavelength, 349 nm; gradient elution, 0–8 min: 80% FA, 8–12 min: 80–60% FA, 12–15 min: 60–50% FA, 15–22 min: 50–80% FA, 22–25 min: 80% FA.

MS conditions: MS analysis was carried out using a Waters API Quattro Micro 2695 LC–MS system equipped with a Waters alliance 2690 pump and an AS 1000 autosampler and UV 1000 variable-wave length UV detector (Thermo Separation Products, FL, USA). Separation was achieved gradiently at room temperature on Phenomenex Gemini C18, 2.0 mm × 250 mm, 5 μm column. The mobile phase consisted of 0.1% formic acid (FA)–acetonitrile (0 min: 95% FA; 24 min: 0% FA; 24, 10 min: 95% FA; 31 min: 95% FA) and was pumped at a flow rate of 0.2 ml/min. The injection volume was 10 μl and the column elution was monitored on-line with UV detection at 350 nm prior to MS detection. MS settings: ESI-, scanning from *m*/*z* 200 to 1200, capillary voltage 3.5 kV, source 100 °C and desolvation *T* (°C): 350 °C. The MS parameters for each flavonoid were optimized by direct infusion of a mixture of the relevant flavonoids into the source. Luteolin and luteolin-7-0-glucoside (Fig. 1) were used as standards in this chromatographical analysis. The correlation coefficient for all calibration curves was ≥0.995. The limit of quantification (LOQ) was 7.5 ng on column. Day-to-day and within-day variations for flavonoid standard samples from all matrices were <5%.

2.9. Data analysis

All data are presented as mean ± SD. Differences between mean values carried out by a one-way analysis of variance (ANOVA) or Student's *t* test and were considered significant at $p < 0.05$. The data obtained with the HPLC assay were transferred to GraphPad Prism-4 program, which was used to plot the standard curves and to obtain the interpolated values using least squares regression analysis.

The permeability (apparent permeability coefficient, cm/s) of each treatment was calculated according to the following equation:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{60AC_0}$$

where dQ/dt is the permeability rate (amount of substance permeated per second), A is the surface area of the membrane (cm^2), and C_0 is the initial concentration of the substance in the donor compartment (μg/ml) (Pong et al., 2005; Zhang et al., 2006).

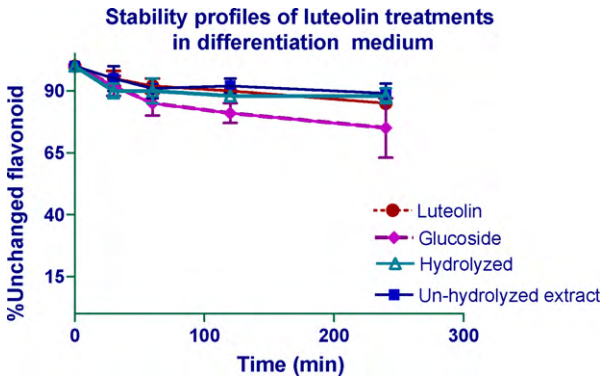

Fig. 3. Stability profiles of luteolin treatments in differentiation medium. Luteolin aglycone, luteolin-7-0-glucoside, un-hydrolyzed and hydrolyzed *Artemisia afra* extracts were kept in DM at 37 °C (5 μg/ml; $n = 3$). Plant extracts were measured in terms of their equivalent concentration of luteolin aglycone.

3. Results

3.1. Luteolin aglycone and luteolin-7-0-glucoside levels in Artemisia afra extract

In this study the freeze-dried aqueous extract of *Artemisia afra* was tested and its luteolin aglycone and luteolin-7-0-glucoside levels were determined before its use in the uptake study. The un-hydrolyzed and acid-hydrolyzed *Artemisia afra* aqueous extract contained 1.06 ± 0.15 and 2.9 ± 0.13 μg/mg of luteolin aglycone, respectively, while the total luteolin glycoside level was 1.9 ± 0.11 μg/mg and luteolin-7-0-glucoside 0.32 ± 0.08 μg/mg. The *Artemisia afra* extract thus contained about one third (35.5%) of its total luteolin in aglycone form and two thirds (64.3%) in glycoside form of which about one sixth (11%) represented luteolin-7-0-glucoside.

3.2. Stability profiles of luteolin preparations in differentiation medium

The percentage of the unchanged luteolin aglycone or its-7-0-glucoside in pure solutions or in un-hydrolyzed or acid-hydrolyzed *Artemisia afra* aqueous extract solutions in the differentiation medium (DM) kept at 37 °C are shown in Fig. 3. The percentage recovery of all the luteolin forms in DM decreased with incubation time. The percentage of luteolin-7-0-glucoside recovered was slightly lower (75 ± 12%), but not significantly different to that of the rest of treatments (i.e. 85 ± 2%, 89 ± 4% and 88 ± 3% for luteolin aglycone and un-hydrolyzed and acid-hydrolyzed *Artemisia afra* aqueous extracts that remained in DM after 240 min, respectively, indicating reasonable stability of all the treatments in this medium.

3.3. Effect of luteolin and Artemisia afra aqueous extract on Caco-2 cells viability

The incubation of Caco-2 cells with different doses of luteolin aglycone and *Artemisia afra* aqueous extract led to a reduction of cell viability as measured by flow cytometric analysis with EC_{50} values of 22 μg/ml and >30 μg/ml for the *Artemisia afra* extract and luteolin, respectively (Fig. 4). The reduction in cell viability was dose-dependent in both treatments, and the cells were more affected by the *Artemisia afra* extract, but a significant reduction (relative to the control) of viability was only recorded with plant

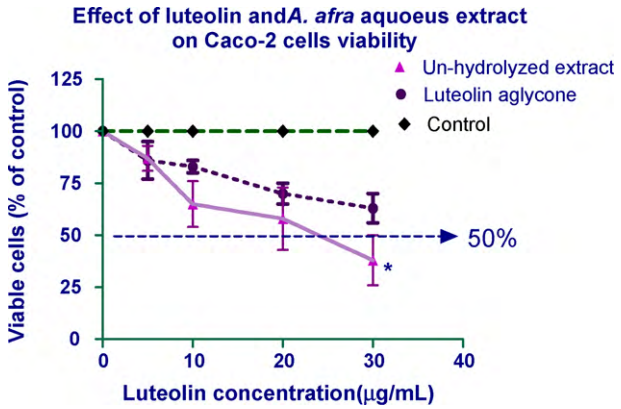

Fig. 4. Effect of luteolin and *Artemisia afra* aqueous extract on Caco-2 cells viability. Caco-2 cells were incubated with luteolin and plant aqueous extract at different concentrations for 150 min. Results are expressed as percentage of control value ± SD ($n = 3$, $^*p < 0.05$ compared to the control).

extract concentrations at 30 μg/ml. In Fig. 5, the effect of the treatments on cell viability are shown with normal cells remaining in the first decade (M1) and the percentage indicating the number of normal cells in the population and the second decade (M2) representing the dead cells with the percentage of cells that died within the population. In the untreated sample the percentage of viable cells was >90% (Fig. 5A), while cells treated with camptothecin (2 μM) showed more than 84% of apoptotic cell death (Fig. 5B). The treated cells showed a dose-dependent decrease in viability. Caco-2 cells treated with 5, 10, 20 and 30 μg/ml of luteolin aglycone showed decreasing viability with less than 31% of apoptotic cell death at 30 μg/ml (Fig. 5C and E). Cells treated with the luteolin aglycone equivalent doses of plant extract forms also showed decreasing viability with only 55 and 35% viable cells at 20 and 30 μg/ml (Fig. 5D and F), respectively. Collectively, the data suggests that both luteolin aglycone and *Artemisia afra* aqueous extract doses of 5, 10 and 20 μg/ml were fairly well tolerated by the Caco-2 cells (>55% viable at 20 μg/ml) over the 150 min experiment period.

3.4. Uptake and metabolism of luteolin aglycone, luteolin-7-0-glucoside and un-hydrolyzed and acid-hydrolyzed *Artemisia afra* aqueous extracts in Caco-2 cells

After incubation of Caco-2 cell monolayers with 5, 10 or 20 μg/ml of luteolin aglycone, its-7-0-glucoside, un-hydrolyzed or acid-hydrolyzed *Artemisia afra* extracts loaded at the apical side, the levels of luteolin and luteolin-7-0-glucoside were measured in both apical and basolateral compartments. Fig. 6 depicts the rate of

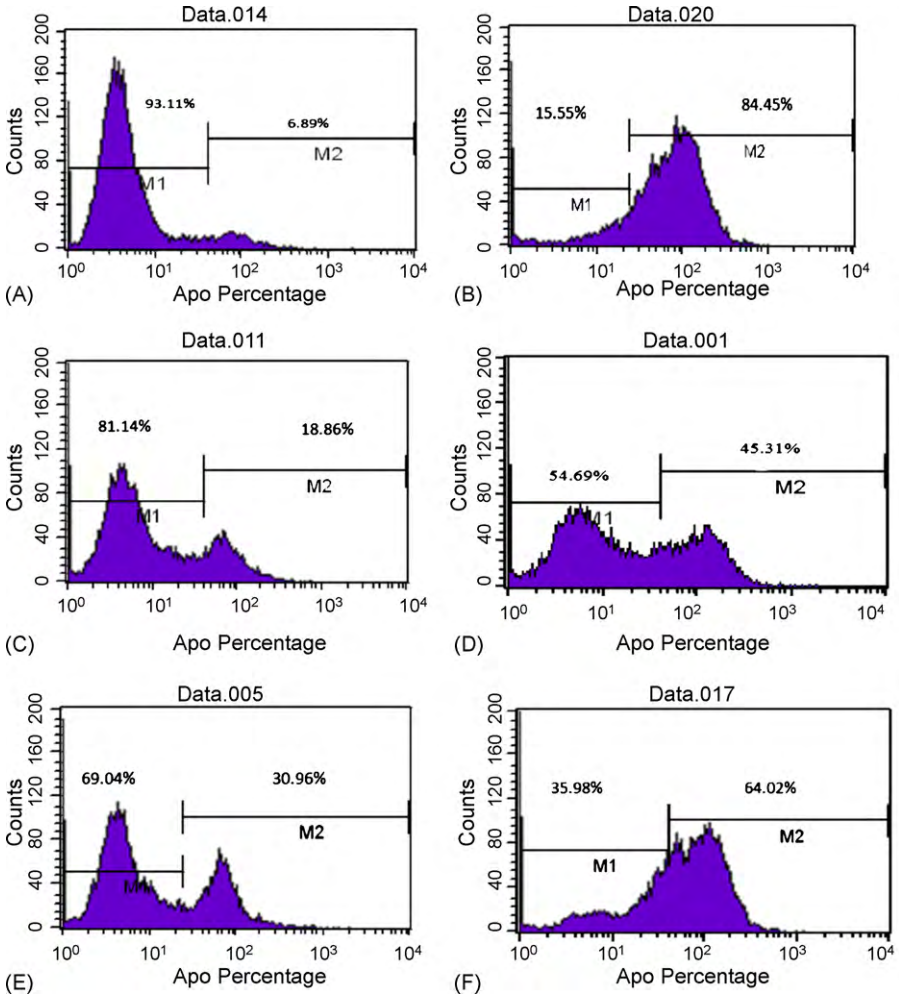

Fig. 5. Histograms depicting the effect of luteolin and *Artemisia afra* aqueous extract in the Caco-2 cells. Cells were seeded in 24-well tissue culture plates, incubated with luteolin aglycone or plant aqueous extract or camptothecin (2 μM), stained with annexin-V PE dye and subjected to flow cytometry. (A) Untreated cells (negative control), (B) cells treated with camptothecin (positive control), (C) cells treated with luteolin aglycone (20 μg/ml), (D) cells treated with *Artemisia afra* (equivalent of 20 μg/ml), (E) cells treated with 30 μg/ml of luteolin aglycone and (D) cells treated with its equivalent in extract form. M1 and M2 represent the percentages of viable cells and apoptotic (non-viable) cells, respectively.

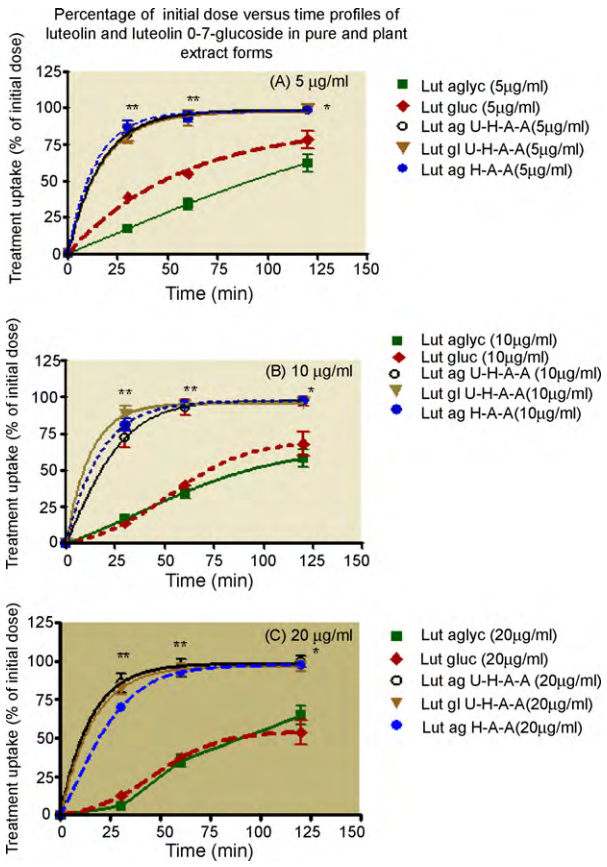

Fig. 6. Apical uptake of luteolin and luteolin glucoside by Caco-2 cells as function of time. Caco-2 cells were exposed to concentrations of 5 μg/ml (A), 10 μg/ml (B) and 20 μg/ml (C) of either luteolin aglycone (Lut aglyc) or luteolin-0-7-glucoside (Lut glu) or un-hydrolyzed (Lut ag U-H-A-A; Lut gl U-H-A-A) and hydrolyzed (Lut ag H-A-A) *Artemisia afra* extract containing the equivalent level of luteolin in the apical compartment. Uptake is expressed as a percentage of the initial concentration and the curves represent mean values of three datasets (**highly significantly different, $p < 0.01$; *significantly different, $p < 0.05$).

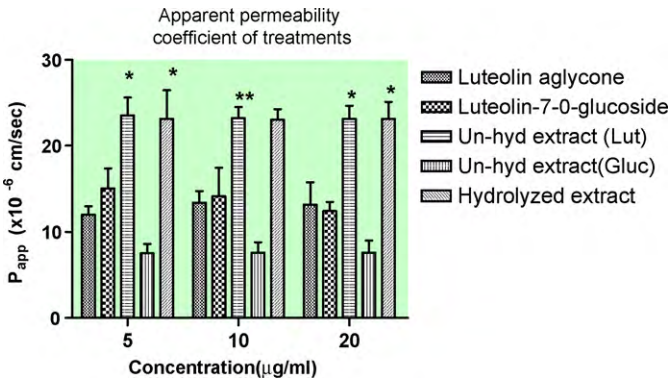

Fig. 7. The apparent apical to the basolateral permeability coefficient (P_{app}) of luteolin aglycone and luteolin-7-0-glucoside administered in pure, and un-hydrolyzed and acid-hydrolyzed *Artemisia afra* extract forms in Caco-2 cells at 3 different concentrations (5, 10 and 20 μg/ml). The histograms represent the mean values of three datasets. *Significantly different ($p < 0.05$) from luteolin aglycone.

disappearance or uptake of luteolin aglycone and its-7-0-glucoside in the apical compartment.

There were slow and low uptake of luteolin aglycone and luteolin-7-0-glucoside from the pure solutions at all 3 doses. Less than 17 and <30% of luteolin aglycone and luteolin-7-0-glucoside, respectively, were taken up by the Caco-2 cells over the first 30 min and this increased to around 60% at 120 min. Compared to the pure solutions, the uptake of luteolin aglycone and luteolin-0-7-glucoside from the un-hydrolyzed and luteolin aglycone from the acid-hydrolyzed *Artemisia afra* aqueous extracts was significantly faster (initial rate) and quantitatively higher (i.e. >77% vs. 17% of the initial doses, $p < 0.05$) over the first 30 min, and essentially complete (~100%) within 60 min. After 120 min 100% of all the flavonoids in the *Artemisia afra* extract solutions were absorbed compared to about 60% of that contained in the pure solutions. The profiles for the luteolin aglycone and its-7-0-glucoside uptake from the un-hydrolyzed and acid-hydrolyzed extracts, although distinctly different from that for the pure solutions, were nevertheless similar to each other over the three doses used. Regardless of the *Artemisia afra* aqueous extract form i.e. whether hydrolyzed or un-hydrolyzed, both forms of luteolin (i.e. aglycone and glucoside) were better taken up by Caco-2 cells from the extract than from the pure solutions.

The apparent apical to the basolateral permeability coefficient (P_{app}) for luteolin aglycone or its-7-0-glucoside in pure solutions or in un-hydrolyzed or acid-hydrolyzed *Artemisia afra* aqueous extract solutions are summarized in Fig. 7. The P_{app} for each flavonoid, whether from pure or extract solutions, essentially remained unchanged over the 5–20 μg/ml concentration range used. However, at 120 min the P_{app} coefficients for luteolin aglycone from the un-hydrolyzed and acid-hydrolyzed plant extract solutions, were significantly higher (i.e. $>23 \times 10^{-6}$ cm/s, $p < 0.05$) than that for this flavonoid from the pure solution (i.e. $<13.3 \times 10^{-6}$ cm/s). The P_{app} coefficients for the luteolin-7-0-glucoside from pure solution, and for that from un-hydrolyzed *Artemisia afra* aqueous extract was higher but the doses were not equivalent (i.e. 1 μg/ml in pure vs. 0.32 μg/ml in *Artemisia afra* extract solution). When adjustment is made for the different dose levels, the P_{app} for the glucoside from pure vs. un-hydrolyzed extract solution were $<15.7 \times 10^{-6}$ cm/s vs. $>23.8 \times 10^{-6}$ cm/s, respectively and significantly different ($p < 0.05$), suggesting that even the uptake of the glucoside is better from the extract than the pure solution form.

The percentage recoveries of both luteolin and its-7-0-glucoside from pure and plant extract forms are shown in Table 1. After 2 h

Table 1
Recovery of luteolin aglycone and luteolin-7-0-glucoside in the basolateral compartment after apical administration of various treatments to the Caco-2 cells.

Treatment	Recovery (%)						
	5[a]		10[a]		20[a]		Control[a]
Luteolin aglycone	2.1 ± 0.3	–	4.6 ± 2.3	–	8.2 ± 3.1	–	43.2 ± 6.5
Luteolin-7-0-glucoside	n/q	–	n/q	–	2.8 ± 1.3	–	42.4 ± 2.9
Un-hydrolyzed extract (Lut)	n/q	–	n/q	–	n/q	–	43.1 ± 3.1
Un-hydrolyzed extract (Glu)	n/q	–	n/q	–	n/q	–	39.2 ± 2.1
hydrolyzed extract (Lut)	n/q	–	n/q	–	n/q	–	44.0 ± 4.2

Each value is the percentage of the initial treatment dose (i.e. 5, 10 and 20 μg/ml). Data are expressed in mean ±SD ($n = 3$); Lut = luteolin aglycone; Glu = luteolin glucoside as in extract; n/q = not quantifiable. In the control, inserts without Caco-2 cells were incubated with 10 μg/ml luteolin aglycone or luteolin-7-glucoside or its equivalents in extract.

[a] Dose (mg/ml).

Table 2
Negative ion MS–MS fragmentation patterns and identification of metabolites after 2 h incubation of Caco-2 cells with luteolin aglycone, luteolin-7-0-glucoside and *Artemisia afra* aqueous extract.

Compound	$[M-H]^-$ (m/z)	MS^2 fragment ions
Luteolin	285	
Luteolin-7-0-glucoside	477	285 ($[M-H]^-$-Glc)
Luteolin monoglucuronide[a]	461	285 ($[M-H]^-$-GlcUA)
Luteolin sulfate[a]	365	285 ($[M-H]^-$-SO_3)
Luteolin monoglucuronide sulfate[b]	541	461($[M-H]^-$-SO_3), 365 ($[M-H]^-$-GlcUA), 285($[M-H]^-$-SO_3-GlcUA)
Methylated luteolin sulfate[b]	379	299 ($[M-H]^-$-SO_3)
Unidentified compound	447	447–282.7–152.5

Unidentified compound was only found in samples incubated with un-hydrolyzed plant extract (no much could be say about this before further studies); free luteolin and free luteolin-7-0-glucoside detected only in the basolateral side of the Caco-2 monolayer treated with pure flavonoids; $[M-H]^-$ = molecular ion; m/z = mass to charge ration; GlcUA = glucuronyl unit; Glc = glucosyl unit.

[a] Metabolites found on both apical and basolateral sides.

[b] Metabolites found on the basolateral side.

incubation with the plant extract, in all three concentrations, neither luteolin nor its glucoside could be recovered in quantifiable levels on the basolateral side. Incubation with the pure flavonoid solutions resulted in 2–8.5% recovery of the 5–20 μg/ml concentrations of luteolin aglycone and only 2.8% of its-7-0-glucoside after incubation at the high concentration (i.e. 20 μg/ml). Very little of the luteolin and luteolin-7-0-glucoside taken up by the Caco-2 cells thus ends up unchanged on the basolateral side.

3.5. Metabolite profile after incubation of luteolin aglycone and luteolin-7-0-glucoside in pure and plant extract forms in Caco-2 cells

After 2 h incubation of Caco-2 cells with luteolin aglycone or luteolin-7-0-glucoside or *Artemisia afra* un-hydrolyzed and acid-hydrolyzed aqueous extracts, more than 90% of the parent compounds appeared to have been metabolized. The metabolite profiles after exposure of the cells to the pure flavonoid and plant extract solutions were similar, except that unchanged luteolin and luteolin-7-0-glucoside were also detected on the basolateral sides of cells exposed to the corresponding pure parent compound solutions. This indicates that unchanged luteolin and its-7-0-glucoside are transported across Caco-2 cells. Recorded metabolites included sulfated, monoglucuronide, sulfated monoglucuronide and sulfated methylated luteolin found in the apical and/or basolateral compartments (Table 2). After exposure of the cells to both pure flavonoid and *Artemisia afra* extract solutions, luteolin monoglucuronide and sulfate were found in both the apical and the basolateral compartments while the sulfated monoglucuronide and the sulfated methylated luteolin were only traced to the basolateral compartment. The luteolin monoglucuronide appeared to be the major metabolite in the Caco-2 cells. In addition, one unidentified compound having the same molecular mass as luteolin glucoside but different retention times and which only appeared once the luteolin and its-7-0-glucoside disappeared, was found in samples incubated with the un-hydrolyzed plant extract. No unchanged luteolin could be detected in cells treated with luteolin-7-0-glucoside.

4. Discussion

This study investigated the uptake and metabolism of the flavone luteolin, in aglycone and glucoside forms, when introduced as pure solutions or aqueous *Artemisia afra* plant extract in human intestinal epithelial Caco-2 cells. The objective was to ascertain the effect that the structure (i.e. whether aglycone or glycoside) of the flavonoid and the plant matrix may have on the intestinal uptake and metabolism of flavonoids from a traditional plant medicine.

Luteolin is a major flavonoid constituent of *Artemisia afra* (Mukinda, 2005) and a primary candidate that might contribute to the claimed *in vivo* protective effects of this medicinal plant. However, for the luteolin in *Artemisia afra* to contribute to the *in vivo* effects produced by this plant, this flavone must be effectively absorbed into the blood stream and reach the active site. As for other plants (Shimoi et al., 1998), luteolin in *Artemisia afra* is in the aglycone and glycosylated forms, each of which may have different uptake and metabolic profiles. Little is, however, known about the uptake and metabolism of luteolin and its glycosylated forms, especially from plant matrix. Shimoi et al., using *in vivo* and everted intestinal rat models, showed that the plasma of rats administered luteolin contained free luteolin (aglycone form), glucuronide and sulfate conjugates of unchanged luteolin and o-methyl luteolin (diosmetin or chrysoeryol) (Shimoi et al., 1998). Clearly, luteolin administered in pure aglycone form is absorbed, but substantially converted to conjugates on passing through the intestinal mucosa with the main metabolite appearing to be a monoglucuronide. On the other hand, the luteolin-7-0-β-glucoside was hardly absorbed by itself, but was first and mainly hydrolyzed to luteolin which was then absorbed and converted to glucuronides (Shimoi et al., 1998). Wittemer et al. investigating the bioavailability of luteolin glycosides contained in Artichoke leaf extracts in humans, found no free luteolin or luteolin-7-0-glucoside in the plasma or urine and very small amounts (1.72–1.99%) of luteolin conjugates in the 24 h urine, but evidence of sulfate or glucuronide conjugates (i.e. phase II metabolites) in the plasma (Wittemer et al., 2005). The question regarding which form of luteolin (i.e. the aglycone or glycoside form) is better absorbed and metabolized thus was unanswered.

Artemisia afra is mainly used in a traditional tea form (decoction) (Van Wyk et al., 2000), in which the levels and proportion of free and glycosylated luteolin can be expected to vary depending on the quality of the plant material, method of preparation of the decoction and its storage. The variation in free and glycosylated luteolin levels in turn will affect the bioavailability of the luteolin from such preparations especially if the free and glycosylated luteolin have different uptake and metabolic pathways. In this study, a freeze-dried aqueous extract of *Artemisia afra* was investigated as a representation of the tea dosage form. This aqueous extract contained 35.5% of its total luteolin content in aglycone form and 64.3% in acid-labile conjugated form of which 11% was luteolin-7-0-glucoside. The luteolin aglycone and glucoside from this *Artemisia afra* extract (and pure solutions) were fairly stable in the medium used. Also, the *Artemisia afra* extract and pure luteolin solutions, in the doses used, did not affect the viability of the Caco-2 cells.

To establish the effect that the plant matrix may have on the *in vitro* uptake and metabolism of the luteolin aglycone and luteolin-7-0-glucoside found in *Artemisia afra*, the uptake of pure solutions of the two flavonoids and the un-hydrolyzed and acid-hydrolyzed freeze-dried aqueous extracts of *Artemisia afra* were compared. From pure solutions administered on apical side, the luteolin aglycone was effectively taken up by Caco-2 cells and its permeability coefficient ($P_{app} = 13.3 \times 10^{-6}$ cm/s) appeared unaffected by concentration variations. Yee (1997) suggested that, in general, compounds with $P_{app} < 1 \times 10^{-6}$ cm/s, $1–10 \times 10^{-6}$ cm/s, and $>10 \times 10^{-6}$ cm/s will be poorly (0–20%), moderately (20–70%) and well (70–100%) absorbed in the gut. The results of the present study thus suggested that luteolin aglycone in pure solution would be well absorbed in the gut. Indeed free luteolin has been found in the plasma of rats after the oral administration of aglycone (Shimoi et al., 1998). The P_{app} value for luteolin aglycone (pure solution) in this study (i.e. in Caco-2 cells) was twice that found by Pong et al. (2005), but this may be due to the differences in method of culture (i.e. 3 days vs. 21 days culture period), passage number and trans-

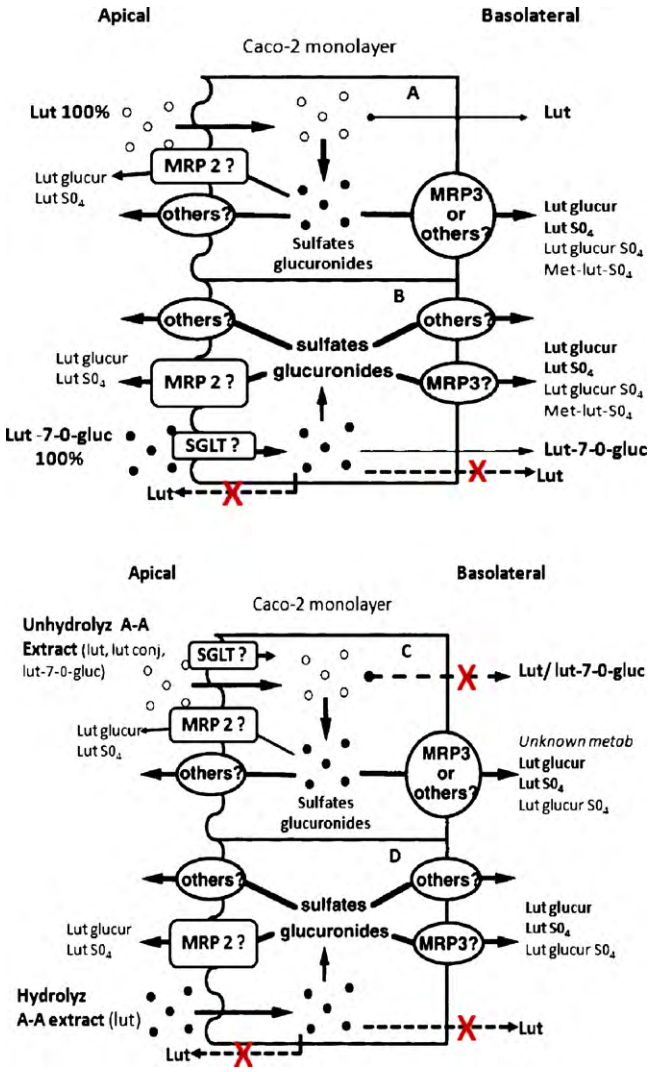

Fig. 8. Hypothetical schematic representation of cell disposition pathways of luteolin and its glucoside in pure and plant extract forms. Synergistic role of conjugative enzymes and efflux transporters for organic anions. After being taken up by enterocytes, some of the flavonoids are metabolized to form the glucuronide and sulfated conjugates. These conjugative metabolites are excreted from enterocytes via transporters for organic anions (such as multidrug resistance-associated protein (MRP) 2 and 3, and other transporters) (adapted according to Suzuki and Sugiyama, 2000). A: Lut = Luteolin aglygone 100%; B: luteolin-7-0-glucoside 100%; C: un-hydrolyzed *Artemisia afra* aqueous extract; D: acid-hydrolyzed *Artemisia afra* extract.

port medium used. In addition to the unchanged aglycone, luteolin monoglucuronide and sulfate were found on both the apical and the basolateral sides, and traces of the sulfated monoglucuronide and sulfated methylated luteolin only on the basolateral side, after apical introduction of the pure aglycone (Fig. 8A). Pong et al. (2005) similarly found glucuronides of luteolin and sulfated luteolin on both apical and basolateral sides and speculated that both apical transporters, such as MRP2, and basolateral transporters, such as MRP3, may be involved in the transport of the luteolin conjugates. Apparently, the nucleophilicity of the 7-OH position of luteolin favoured its conjugation with glucuronic acid (Pong et al., 2005). Taken together it appears that because of its lipophilicity, luteolin is taken up into Caco-2 cells by simple diffusion and/or active transport, then effluxed from the cell, most likely by transporter such as MRP1 or MRP3, and metabolized in the intestinal cell into a variety of conjugates that in turn are actively transported (i.e. effluxed via MRP2 and MRP3) into apical and basolateral sides, respectively, with the basolateral transporters probably being more active (Suzuki and Sugiyama, 2000; Schutte, 2007) (see Fig. 8A). [The phase II conjugations of the luteolin and subsequent secretion of the luteolin conjugates from the Caco-2 cell are the most important features in this scenario.]

When administered as a pure solution on the apical side, luteolin-0-7-glucoside was also well absorbed with a permeability coefficient (P_{app}) slightly higher ($<15.7 \times 10^{-6}$ cm/s) than that for the aglycone, and independent of the concentration variations. The metabolite profile was similar to that found for the luteolin aglycone, except that luteolin-0-7-glucoside (especially at high i.e. 20 μg/ml dose) but no free luteolin aglycone was detected on the basolateral side. This, firstly, suggests that the unchanged parent luteolin-0-7-glucoside was transported across Caco-2 cell monolayer as a glucoside. Secondly, the luteolin-0-7-glucoside was hydrolyzed to the aglycone which in turn was quickly converted into metabolites. The uptake of the luteolin glucoside, from pure solution, has been demonstrated in animals (Shimoi et al., 1998; Wittemer et al., 2005) but not yet in Caco-2 cells. Liu and Hu (2002) found that apigenin-7-0-glucoside (i.e. apigetrin) was transported across the Caco-2 cells but at much lower rates than the aglycone. Walgren et al. (1998) found that no apical to basolateral transport of quercetin-4-glucoside by Caco-2 cells, but such transport of quercetin-3, 4′-diglucoside, albeit also at rather low rate. Although in our study the luteolin-7-0-glycoside transport was as efficient as for aglycone, it is generally accepted that the intestinal transport of flavonoid glucosides occurs via the sodium-dependent glucose transporter (SGLT) and that the efficiency of such transport was dramatically suppressed by efflux (of at least some flavonoid glycosides) via the apical multidrug resistance-associated protein 2 (MRP2) (Walle, 2004) and/or quick metabolism (Liu and Hu, 2002). Among flavonoid glycosides, differences in their affinities for transporters systems and susceptibilities to different enzymes, which in turn is dependent on the position, number and type of sugars in the conjugate (Manach et al., 2004), will thus determine the extent to which their transport across Caco-2 cells occur. According Liu and Hu (2002) slow passive diffusion, poor uptake via SGLT1, and the presence of efflux carriers for glycosides render the intestinal hydrolysis as the critical first step in the intestinal disposition of glycosides. Overall, our findings suggest that luteolin-0-7-glucoside, from pure solutions, is taken up into Caco-2 cells by active transport, very likely via the SGLT transporter, efficiently conjugated intracellularly and the phase II metabolites effluxed from the cell by more transporter systems such as MRP3 on basolateral and MRP2 on apical side (Fig. 8B). Because of the similarity in the P_{app} values for luteolin-7-0-glucoside and the luteolin aglycone it is unlikely that the structure (aglycone vs. glycoside) has a significant impact on luteolin uptake in Caco-2 cells. The mechanism for the basolateral secretion of the glucoside is however unclear at this stage, but as was the case for the aglycone, phase II metabolism appears to have a significant impact on the disposition of the flavonoid glucoside in the Caco-2 cell.

The main purpose of the current study was however to ascertain if and how the matrix of plant aqueous extract would influence the transport of luteolin and its-7-0-glucoside. Aqueous extract of *Artemisia afra* that contains luteolin, luteolin-7-0-glucoside and other luteolin conjugates and the acid-hydrolyzed aqueous extract that only contained the aglycone were tested. After incubation of Caco-2 cells with these extracts of *Artemisia afra* on the apical side, luteolin and its-7-0-glucoside were, with P_{app} values $>23 \times 10^{-6}$ cm/s vs. $<15.7 \times 10^{-6}$ cm/s, taken up significantly better from the extracts than their pure solutions (Fig. 6). Although a profile of metabolites that was similar to that seen after the exposure of the cells to the pure solutions was obtained, no free luteolin aglycone or glucoside was detectable on the basolateral side and more than 90% of both flavonoid forms were quickly metabolized. Collectively, this suggests that luteolin and its-7-0-glucoside from

Artemisia afra aqueous extracts had a more efficient metabolism in the Caco-2 cells than that in pure solutions. Luteolin and its-7-0-glucoside in *Artemisia afra* aqueous extract is thus taken up by, metabolized in and the metabolites effluxed from Caco-2 cells in a qualitatively similar manner to that of the pure solutions but their uptake appears to be quantitatively more efficient and metabolism more extensive (Fig. 8C). As was the case for the pure solutions the P_{app} values for the aglycone vs. the glucoside from the extract were equal confirming that the structure of the luteolin did not significantly affect the flavonoid's uptake in Caco-2 cells, more likely something associated with plant matrix itself may be responsible for the enhanced uptake of both luteolin flavonoid forms from the *Artemisia afra* extract.

When acid-hydrolyzed extract of *Artemisia afra*, which contains only aglycone luteolin, was incubated with Caco-2 cells, the uptake (P_{app} value) was similar to that for aglycone from the un-hydrolyzed extract and also greater than that for the pure aglycone solution. The metabolite profile was similar to that obtained with the un-hydrolyzed extract and again no free luteolin was found in the basolateral side. Clearly luteolin was still qualitatively and quantitatively taken up, metabolized and the metabolites effluxed in this modified matrix (Fig. 8D) in the same way as in the unchanged matrix (un-hydrolyzed extract). Significantly it was, like the un-hydrolyzed extract, also quantitatively better taken up by the Caco-2 cells than the pure aglycone (i.e. in the absence of plant matrix). In *Artemisia afra* the plant matrix, whether acid-hydrolyzed or not, therefore appears to enhance the apical to basolateral transport of the luteolin aglycone in Caco-2 cells. How this enhancement is brought about is unclear at this stage. It is possible that some constituents in the matrix might in theory induce inhibition of the apical efflux transporters (e.g. MRPs) and enhance metabolism of flavonoids while the basolateral transporters remain more active in excretion of conjugates.

Finally, luteolin glucoside is seen as a water-soluble flavonoid and its aglycone as highly lipophilic flavonoid (Walle, 2004). Since, both forms from the plant extracts were better taken up in Caco-2 cells than from their pure solutions, two pathways were thus considered as the possible routes by which both luteolin and its-7-0-glucoside could be transported across the Caco-2 monolayers. Firstly, by the transcellular route: the flavonoids are transported through the cell membrane and undergo prior hydrolysis then metabolic conversion. Secondly, by the paracellular route; flavonoids cross through the tight junctions bridging the intercellular spaces between the cells (Murota et al., 2002). However, the effect of flavonoids on the transepithelial electrical resistance (TEER) was measured to clarify the mechanism of uptake of luteolin and its-7-0-glucoside. The TEER values did not vary significantly in all treatments before and after the study period, suggesting that the flavonoids from the plant extracts were taken up through the cell membrane and transported with rapid hydrolysis followed by a quick metabolic conversion. In other words, the flavonoids were readily transported through the Caco-2 monolayer, presumably because of their affinity toward the lipid bilayer of the cell surface. The glucoside was most likely hydrolyzed to its aglycone during absorption. This is supported by the *in vitro* study reporting that the structurally related apigenin-7-0-glucoside was hydrolyzed by a cell free extract of human small intestine (Wittemer et al., 2005). In addition, the enzymes that are able to hydrolyze flavonoid glucosides are reported to be located in the cells (cytosolic beta-glucosidase, CBG) and on the apical membrane (lactase-phlorizin hydrolase, LHP) (Wittemer et al., 2005). Hence, flavonoid glucoside (i.e. luteolin-7-0-glucoside) may be cleaved by LHP, after which the aglycone may diffuse passively into the cell (Day et al., 2000), or alternatively, the flavonoids may enter the cells as an intact glucoside by sodium-dependent glucose transporter (SGTL1) and then be cleaved inside the cell by CBG (Wittemer et al., 2005). Secondly, the luteolin aglycone (and the newly formed from hydrolysis) were quickly and more efficiently converted to its glucuronides/sulfates after absorption by the intracellular conjugation enzymes (i.e. glucuronosyltransferase and others) (Murota et al., 2002; Pang, 2003).

Overall, the results of the present study indicate that, after incubation with Caco-2 cells, luteolin in the *Artemisia afra* aqueous extract, regardless of its form (i.e. whether aglycone and 7-0-glucoside), is taken up better and is more efficiently metabolized than the aglycone and 7-0-glucoside forms of this flavonoid administered as pure solutions, strongly suggesting that the plant matrix may have a facilitating effect on the uptake and subsequent metabolism of luteolin from *Artemisia afra*. Since the Caco-2 cell model is a fairly good predictive model for human oral absorption, the results obtained should also fairly closely reflect the *in vivo* intestinal uptake and metabolism of luteolin expected when *Artemisia afra* aqueous plant extracts and the traditional tea are given to humans. This means that flavonoid actives from *Artemisia afra* plant extracts and especially traditionally prepared dosage forms may have better bioavailability, and consequently greater *in vivo* potency, than that predicted from studies done using the pure solutions. Because the proportion of glycoside to aglycone form of the luteolin may, due to method of preparation, storage conditions, etc., also vary in plant extracts, significant differences in bioavailability, and thus therapeutic performance, of poorly prepared or stored extracts or traditional dosage forms of *Artemisia afra* can be anticipated. Further studies are however required to specifically check the uptake and metabolism of luteolin in plant form in humans, since some factors (e.g. the enzyme systems that are involved in metabolism of luteolin, etc.) could differ between humans and cell monolayers. In addition, the reason for the enhanced uptake, i.e. whether it is associated with physicochemical properties (e.g. dissolution, etc.) and/or uptake mechanisms and/or effect of other co-existing constituents in plant extracts, etc. should also be established.

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