# Luteolin levels in selected folkloric preparations and the bioavailability of luteolin from *Artemisia afra* aqueous extract in the vervet monkey

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***Abstract***

*In this study, the level of luteolin was determined in various plant materials:* ***Artemisia afra, Mentha longifolia, Hypoestes rosea and Hypoestes triflora*** *by HPLC assay. The high level of luteolin was found in* ***Artemisia afra.*** *The bioavailability of luteolin either in pure or plant form was determined in the vervet monkey using HPLC assay. The peak levels of luteolin were found 30 min after oral administration of pure luteolin and luteolin in plant extract form which presented the best bioavailability of luteolin. In both forms the elimination process of luteolin was fast and similar ($K_e$ = 0.0054 ± 0.0006/min and $T_{½}$ 132.21 ± 15 min.) and the levels of luteolin in urine and faeces samples were low and not significantly different.*

***Résumé***

*Dans cette étude, la concentration de la lutéoline a été déterminée par HPLC dans diverses matières végétales :* ***Artemisis afra, Mentha longifolia, Hypoestes rosea et Hypoestes triflora.*** *La plus grande concentration de la lutéoline a été trouvée dans l'****Artemisis afra****. La biodisponibilité de la lutéoline sous la forme pure ou industrialisée a été déterminée par HPLC chez le singe. Les concentrations maximales de la lutéoline ont été trouvées 30 minutes après administration orale de lutéoline pure et de lutéoline sous la forme d'extrait de plante et c'est cette dernière forme qui a présentée la meilleure biodisponibilité. Pour les deux formes, le processus d'élimination de la lutéoline était rapide et semblable (Ke = 0.0054 ± 0.0006/min et ½ de T = ± 132.21 15 mn) et les concentrations de la lutéoline dans des échantillons d'urine et de selles étaient basses et pas sensiblement différentes.*

## 1. INTRODUCTION

Flavonoids, members of a class of natural polyphenolic compounds, appear to have played a major role in the successful medical treatments of ancient times, and their use has persevered up to now (Pei-Dawn et al., 2002; Shu-Ping and Kuo-Jun, 2004; Rice-Evan, 2004). Flavonoids are polyphenolic compounds found in many plants in which they are often responsible of the coloration of flowers, fruits and leaves. It has been reported that flavonoids are present in *Arthemisia afra*, *Mentha longifolia* (Van Wyk et al., 2000; Harris, 2002; Waithaka, 2004) and they should also be present in *Hypostes rosea* and *Hypostes triflora* in which they could play a major role as active ingredients. Flavonoids are divided into several classes which have different chemical compounds, dietary sources, pharmacology effects and bioavailabilities. Among these classes, we can cite the flavones, a class which contains luteolin as an example.

In this study, the flavone luteolin was investigated in *Artemisia afra* and in *Mentha longifolia*, ones of the most common medicinal plants used in South Africa, and in two Rwandan medecinal plants *Hypoestes rosea* and *Hypoestes triflora*. Luteolin bioavailability in pure form and in plant extract was determined in vervet monkeys. Luteolin (5, 7, 3', 4' tetrahydroxy flavone) is found mostly in glycoside form in many plants, fruits and some honeys, and often acts as pigment (Shimoi et al., 1998; Sampson et al., 2002 and Yao et al., 2004). It is said to have many beneficial properties such as antioxidant, anti-cancer, anti-carcinogenic, anti-inflammatory and hepatoprotector activities, as well as the ability to reduce the biosynthesis of cholesterol (Hoffman, 1995; Arcias et al., 2001; Adzet et al., 1987 and Woodham, 2002). For luteolin-containing plants to display these luteolin-mediated activities, the luteolin (in the plant product) must have the appropriate pharmacokinetic (i.e. absorption, distribution-metabolisation and excretion) charac-teristics and bioavailability (i.e. have an optimal rate and extent of absorption to reach the side of action). There is a very large amount of *in vitro* data on flavonoids, however very few reports of animal studies are available, especially for luteolin (Shimoi et al. 1998; Pei-Dawn et al., 2002). Spencer et al. studied the absorption of luteolin aglycone and monoglycoside in an isolated rat intestine model and found, after treatment with β-glucuro-nidase, at least six different glucuronides in the plasma (Spencer et al., 1999). It

was concluded that the luteolin was mono-glucuronidated at different sites and could possibly also be di- or tri- glucuronidated. Measured levels of the aglycone in the plasma were small relative to the amount of total luteolin glucuronide recovered after intestinal perfusion with luteolin aglycone and its glucuronides. The recoveries of both forms were lower across the ileum relative to the jejunum (Spencer et al., 1999). Shimoi et al. (1998) confirmed that luteolin was converted to glucuronides in intestinal mucosa and luteolin 7-O-β-glucoside was absorbed after hydrolysis to luteolin. They reported that free luteolin, its conjugates and methylated conjugates were present in rat plasma after its oral administration in pure form and that some luteolin could escape the intestinal conjugation and the hepatic sulfation / methylation. Shimoi et al. (1999) investigated the bioavailability of luteolin and reported that luteolin and its conjugates in rat plasma increased to the highest level 15 min or 30 min after oral administration of luteolin in propyleneglycol and decreased. They found similar metabolites in urine and plasma. In the same study, they proposed a scheme of the intestinal absorption and the metabolic fate of luteolin and luteolin 7-O-β-glucoside in which luteolin is mostly absorbed from the duodeno-jejunum, rapidly glucuronidated by UDP-glucuronosyltransferase. Thereafter, luteolin enters the portal vein and reaches the liver where its sulfatation and methylation take place. However, more information is still needed about the pharmacokinetics of luteolin and even less is known about the bioavailability of luteolin from different preparations of the plant medicines.

## 2. MATERIALS AND METHODS

### *2.1. Chemicals*

Luteolin, kaempferol, β-glucuronidase (EC 3.2.1.31 type H-3) and sulfatase (EC 3.1.6.1 type H-2) were purchased from Sigma (St. Louis, USA). Methanol HPLC grade and all other reagents used (analytical grade) were purchased from Merck (Darmstadt, Germany). HPLC column and pre-column were obtained from Anatech (Cape Town, South Africa).

### *2.2. Plant materials*

*Artemisia afra* was purchased from Montague Museum in Montague (Western Cape Province, South Africa). *Mentha longifolia* was obtained from Kirstenbosch National Botanical

Garden (Cape Town, South Africa), *Hypoestes triflora* and *Hypoestes rosea* were collected from the garden of Rwandan Institute of Sciences and Technology i.e. IRST (Butare province, Rwanda).

### 2.3. Animals

The vervet monkeys, *Ceropithecus aethiops*, used in this study were selected from 250 vervet monkeys housed at the Medical Research Council (MRC) Primate Unit, Experimental Biology Programme, Bellville (Western Cape Province, South Africa).

### 2.4. Preparation and analysis of plant extract

The plant material were extracted according to the methods used by the traditional healers i.e. 2 L of boiling distilled water was used to extract 100 g of dried leaves and the mixture boiled for 30 min to facilitate the extraction of the flavonoids.

To determine the presence of flavonoids in the plant extracts the following chemical test (Ikhiri et al., 1992) was used. For each sample 2 g of powdered plant material was dissolved in 20ml of water and heated on a water bath at 80°C for 3 minutes. To 3 ml of the filtrate 3 ml of acid-alcohol (EtOH: $H_2O$: conc. HCl 1:1:1), approximately 20 mg of solid magnesium and about 1ml of amyl alcohol were added and the emergent colour of the solutions observed. The appearance of a rose-orange or violet colour indicated the presence of flavonoids. TLC was also used to characterise flavonoids in the plant extracts. For that, the developed plates in an ascending manner were dried, exposed to ammonia vapour, viewed under normal and ultraviolet light and the spots detected under UV light at 254 and 366 nm and by their behaviour after the ammonia exposure.

The luteolin content of the plant extract was determined by HPLC assay. For this, 25 mg samples of the crude plant extracts dissolved in 200 µl methanol hydrolysed with 4.8ml of hydrochloric acid and aglycone forms of the flavonoids extracted with 4 ml ethyl acetate. The ethyl acetate fraction was evaporated to dryness under a stream of nitrogen gas and residue obtained reconstituted in 200 µl of mobile phase for HPLC essay.

***Preparation of plasma, urine and faeces samples***

**Plasma samples:** The blood samples were collected in vacucontainers containing EDTA anticoagulant 0; 15; 30; 60; 120; 240 and 360 min after oral administration of luteolin either in pure or plant form. Samples were gently mixed and immediately centrifuged at 2500 rpm for ten minutes at 20°C and the plasma was separated from the packed erythrocytes and buffy coat. To 200 µl of plasma samples, 200 µl of a mixture of methanol - 0.5 M HCl (1:1 v/v) and 410 ng of the internal standard, kaempferol were added. The aglycone form of luteolin was extracted with 1 ml of ethyl acetate. For the enzymatic hydrolysis, an amount of 40 units of sulfatase in 1 M sodium acetate buffer (pH 5.0) and 1080 units of β-glucuronidase in potassium phosphate buffer (pH 6.8) were added to each 200 µL of plasma sample. Then, 410 ng of the internal standard was added and all tubes were incubated at 37°C for one hour. Thereafter the tubes were cooled to room temperature, 200 µL of 1 M HCl (to stop the hydrolysis) and 200 µL of acetone (to precipitate proteins) added and luteolin extracted with 2 ml ethyl acetate. The organic phase was evaporated to dryness under a stream of nitrogen, the residue reconstituted in 50 µL of the mobile phase and 20 µL injected onto the HPLC column.

**Urine samples:** After each treatment, the 24 hours urine samples were collected in labelled and clean tubes, centrifuged at 2500 rpm for 10. For acid hydrolysis, to 2 ml of the supernatant collected, 1ml 3 M HCl and 410 ng of the internal standard were added and samples heated at 80°C for 1 hour. An amount of 4 ml ethyl acetate was added to extract luteolin. For the enzymatic hydrolysis, 400 units of sulfatase dissolved in 50 µL of sodium acetate buffer (pH 5.0), 10800 units of β-glucuronidase dissolved in 50 µL of potassium phosphate buffer (pH 6.8) and 410 ng of the internal standard were added to 2 ml of urine. An amount of 4 ml ethyl acetate was used for extraction and after evaporation of the organic phase, the residue was reconstituted in 50 µL of mobile phase and 20 µL injected onto the HPLC column.

**Faeces samples:** Faeces samples of 24 hours were collected from animal after each phase of the study. To each sample, 1 ml chloroform was added to inhibit bacterial growth and then sample freeze-dried. For acid hydrolysis, 1 g of faeces sample (freeze-dried) from each monkey was dissolved in 3 ml of

methanol and tubes centrifuged at 2500 rpm for 10 minutes at room temperature. The supernatant was taken up and evaporated to dryness under nitrogen flow. The residue was reconstituted in 1 ml of methanol, 4 ml 3 M HCl added and samples heated for 1 hour in water-bath maintained already at 80°C. An amount of 4 ml of chloroform was added for extraction of luteolin. For the enzymatic hydrolysis, to the same amount of faeces sample 200 units of sulfatase dissolved in 50 µL of sodium acetate buffer (pH 5.0), 5400 units of β-glucuronidase dissolved in 50 µL of potassium phosphate buffer (pH 6.8) and 410 ng of the internal standard were added. Tubes were vortex-mixed and incubated at 37°C for 1 hour and 2 ml of acetone, 1 ml of 1 M HCl and 4 ml of chloroform was added. The organic phase was evaporated and the residue was reconstituted in 50 µL of the mobile phase and 20 µL injected onto the HPLC column.

### *2.5. HPLC assay of samples*

The mobile phase used consisted of a mixture of methanol and 5% formic acid in water in proportion of 60:40 v/v for the plant extract, urine and faeces samples and 55:45 v/v for the plasma samples. The Luna C18 (2) (250 x 4.60 mm) 5 µm analytical HPLC column was used for this separation and the luteolin was eluted with mobile phase isocratically pumped at 1 ml/min for the plant extract, the plasma and urine and 1.1 ml/min for the faeces samples. The chromatographic separation was done at room temperature (20°C) and the peaks were detected at wavelength of 349 nm. The determination and quantification of luteolin were based on the retention time and luteolin peak area or peak height, respectively, and the diode array UV detector scanning between the wavelengths 168 and 349 nm was used to confirm the identification of the luteolin peak.

## 3. RESULTS

The chemical test and TLC method used indicated the presence of flavonoids in *A.afra, M. longifolia, H. rosea and H. triflora* aqueous extract (results not shown). The high level of luteolin (2548.4 ± 2.24µg/g) was found in the hydrolysed aqueous extract of *A. afra* plant extract (Table 1). The figure 1 and 2 show the HPLC chromatograms of luteolin in monkey plasma after oral administration of luteolin in pure and in plant extract respectively. The concentration of luteolin in monkey plasma

reached the highest level (130.25 ± 22.7 and 258.83 ± 7 ng/ml) 30 min after oral administration of luteolin in pure and plant form respectively (Figure 3).

**Table 1: Concentration of luteolin (µg/25mg plant material mean ± SEM) in plant extracts. Aliquots of plant material were subjected to hydrolysis, extracted with ethyl acetate and assayed by HPLC with UV detection**

| Plant extract | Luteolin concentration µg/25mg plant material ± SEM |
|---|---|
| *Artemisia afra* hydrolysed | 63.71 ± 0.056 |
| Un-hydrolysed | 27.11 ± 0.047 |
| *Hypoestes rosea* | 0.373 ± 0.0422 |
| hydrolysed | 0.112 ± 0.00352 |
| Un-hydrolysed | |
| *Mentha longifolia* | ND |
| (hydrolysed) | |
| *poestes trifolia* (hydrolysed) | ND |

ND = not detectable

The $AUC_{0\text{-}inf}$, and $K_{el}$ after pure luteolin and *A. afra* administration were: 23.104 ± 3.13 and 46.17 ± 2.057 µg/ml.min; and 0.0054 ± 0.0006 and 0.0048 ± 0.0005ng/min, respectively and the point ratios and 90% CI for $AUC_{0\text{-}inf}$ and $C_{max}$ 202.9 % (CI = 175.9 – 233.9%) and 202.2% (CI = 169.5 – 241.1%), respectively. The levels of luteolin in the urine were 129.11 ± 31.13 and 99.46 ± 24.93 ng/ml for the plant extract and pure luteolin, respectively, while that in the faeces samples were 82.56 ± 3.35 and 137.55 ± 2.23 ng/g, respectively. The difference in the urine and faeces levels for the 2 preparations was not significant (p = 0.3190 and 0.4026, respectively). The results given were obtained using acid hydrolysis method; the enzymatic hydrolysis method (results not shown) gave similar results.

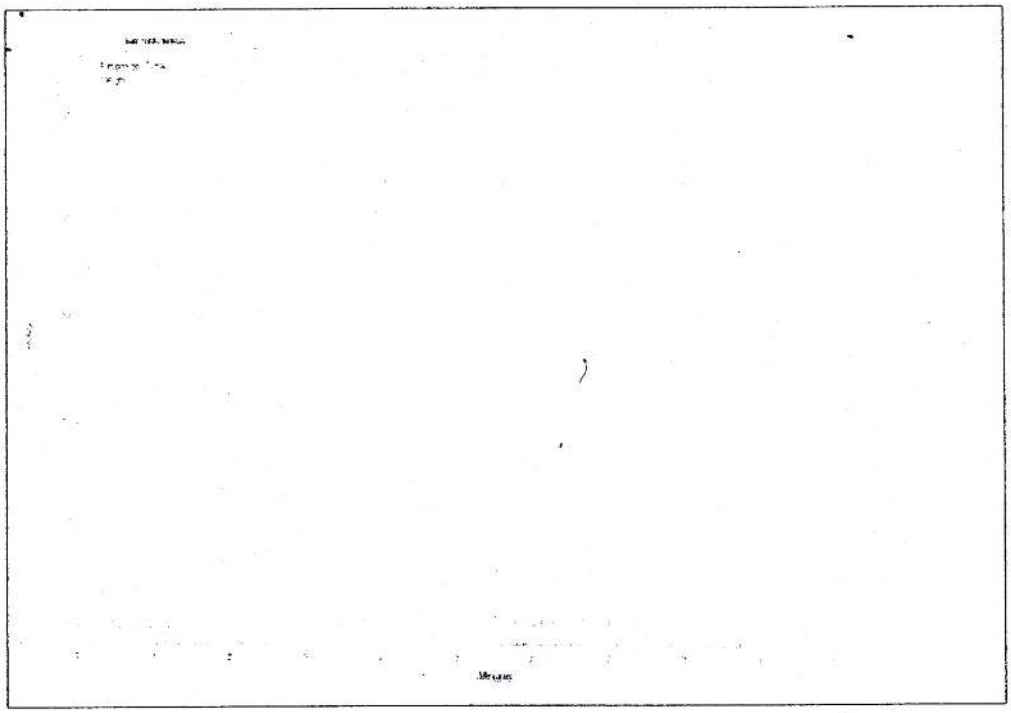

*Figure 1: Representative HPLC chromatogram of monkey plasma sample collected 15 minutes after oral administration of luteolin. The retention time of luteolin (peak L) and the internal standard (peak I.S.) is 7.52 min and 9.62, respectively.*

## 4.Discussion

The peak levels of luteolin were found 30 minutes ($T_{max}$) after oral administration of pure luteolin and luteolin in plant extract, and were 130.52 ± 22.07 ng/ml 258.83 ± 7 ng/ml and 140.8, respectively (Figure 3). Such fast absorption is not entirely unexpected especially since Shimoi et al. (1998), using the rat inverted small intestine model, predicted a $T_{max}$ of 15 min for the intestinal absorption of luteolin in propylene glycol. Shimoi et (1999) found also the same $T_{max}$ (15 or 30 min) in an investigation of luteolin bioavailability. In another study in rats, Liu et al. (1995) found a $T_{max}$ for luteolin of about 1 hour. The slight differences in $T_{max}$ arising from these studies can be explained by the fact that the conditions of administration were different and the animals used (rats and monkeys) were also different.

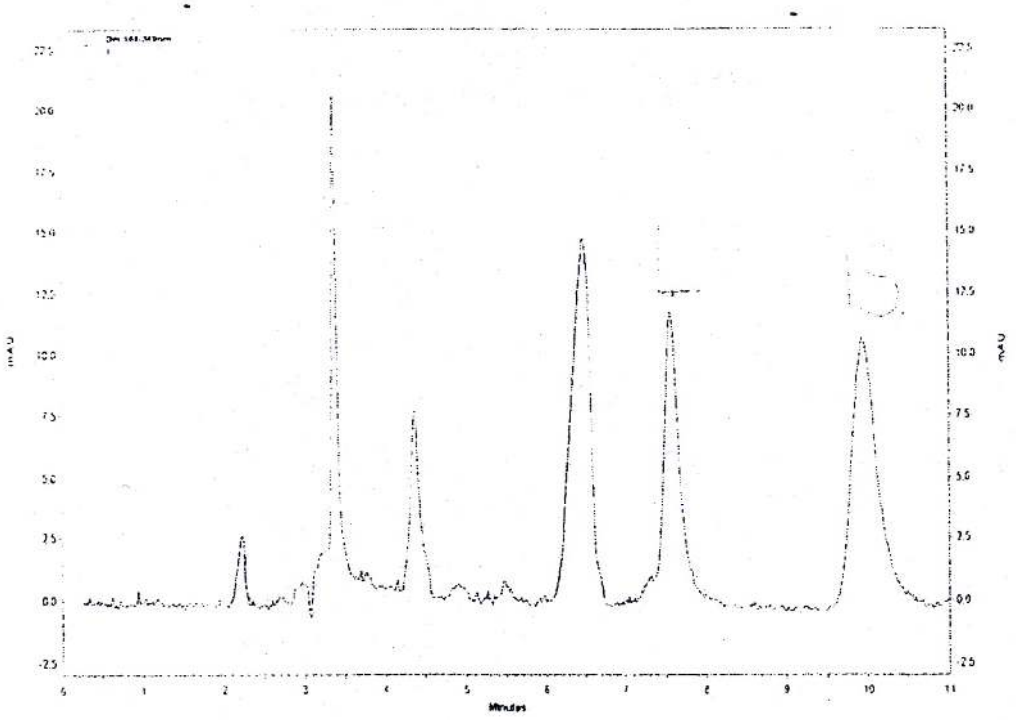


**Figure 2:** *Representative HPLC chromatogram of monkey plasma sample collected 15 min after oral administration of an aqueous extract of A.afra. The retention time of luteolin(peak L) and the internal standard (peak IS)* is 7.55 min and 9.64, respectively.

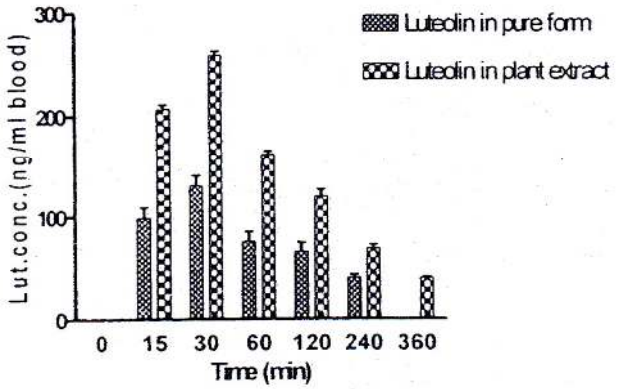


**Figure 3:** *Concentration of luteolin in monkey plasma sample after oral administration of luteolin in pure and in plant form*

For all three parameters ($AUC_{0-360}$, $AUC_{0-\infty}$ and $C_{max}$), the 90% confidence intervals for the ratios were outside the expected range of 80 - 120% for bioequivalence (Table 2). This means clearly that the bioavailability of luteolin in the aqueous extract of *Artemisia afra* was significantly different from that of the pure form. Indeed, the luteolin bioavailability was distinctly better from the aqueous extract of the plant material (Figure 3).

**Table 2:** *Summary of the bioequivalence analysis based on parameters obtained in the vervet monkey (n=6). Each monkey received an equivalent of 1 mg luteolin / kg body weight p.o and 2 ml of blood were collected at different times, the level of luteolin in each sample determined by HPLC assay, the parameters calculated from plasma concentration vs. time data and the point ratios using log-transformed data.*

| Parameters | Luteolin | Plant extract | Point ratio | 90% C.I. |
|---|---|---|---|---|
| $AUC_{(0-360)}$ mean ± SEM (µg/ml.min) | 15.38 ± 4.13 | 37.68 ± 2.41 | 2.37 ± 0.731 | 199.5 – 332.3 |
| $AUC_{(0-\infty)}$ mean ± SEM (µg/ml.min) | 23.104 ± 3.789 | 46.17 ± 2.057 | 2.029 ± 0.255 | 175.9 – 233.9 |
| $C_{max}$ mean ± SEM (ng/ml) | 130.25 ± 22.07 | 258.83 ± 7 | 2.022 ± 0.345 | 169.5 – 241.1 |
| $K_e$ mean ± SEM (/min) | 0.0054 ± 0.0006 | 0.0048 ± 0.0005 | - | - |
| $T_{½}$ mean ± SEM (min) | 132.21 ± 15 | 146.3 ± 15.5 | - | - |

Either the plant contained the luteolin in a more bioavailable form or the *A. afra* extract contained other compounds which may have influenced the absorption characteristics of the luteolin.

Several pharmacokinetic and bioavailability studies have been conducted on luteolin in animals attempt to determine which form of luteolin is absorbed and available in the body (Liu et al., 1995; Shimoi et al., 1998; Spencer et al., 1999). In this study, the comparison of the HPLC chromatograms obtained after enzyme and acid-mediated hydrolysis of the plasma samples collected after the administration of the plant extract clearly gave the same identifiable luteolin peak (i.e. aglycone form) (results not shown). This indicates that some luteolin found in the plasma might have been in glucuronide or sulphate forms as was previously reported (Shimoi et al., 1998). The results of this present study did not however provide any further information about which aglycone or glycoside form of luteolin was possibly absorbed. Nevertheless, this study provided important information about the bioavailability of luteolin in plant extract (*A. afra*).

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