

Effects of Luteolin on Distribution and Metabolism of 2-Aminofluorene in Male Sprague-Dawley Rats

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Abstract. The effects of oral luteolin on the N-acetylation and metabolism of 2-aminofluorene (AF) in vivo were investigated in bladder, blood, colon, kidney, liver, feces, urine, cerebrum, cerebellum and pineal gland from male Sprague-Dawley rats. Major metabolites such as AAF, 1-OH-AAF, 3-OH-AAF, 8-OH-AAF and 9-OH-AAF were found in bladder tissues; AAF, 1-OH-AAF, 5-OH-AAF and 8-OH-AAF were found in blood samples; AAF, 1-OH-AAF, 3-OH-AAF, 5-OH-AAF, 8-OH-AAF and 9-OH-AAF were found in colon tissues; AAF, 1-OH-AAF, 3-OH-AAF and 9-OH-AAF were found in kidney tissues; AAF, 1-OH-AAF, 3-OH-AAF and 8-OH-AAF were found in liver tissues, AAF, 1-OH-AAF, 3-OH-AAF, 5-OH-AAF, 7-OH-AAF, 8-OH-AA and 9-OH-AAF were found in feces and urine samples; AAF, 1-OH-AAF, 3-OH-AAF and 8-OH-AAF were found in cerebrum tissues; AAF, 1-OH-AAF, 3-OH-AAF and 7-OH-AAF were found in cerebellum tissues; but only AF and AAF were found in pineal gland in rats treated with AF (50 mg/kg) for 24 h. Pretreatment of rats with luteolin (30 mg/kg) 24 h prior to the administration of AF (50 mg/kg) and luteolin given with AF concomitantly led to a decrease in the amounts of 3-OH-

AAF and 9-OH-AAF and an increase in the amounts of 1-OH-AAF and 8-OH-AAF in bladder tissues. In blood samples, there were significant decreases of AAF, 1-OH-AAF and 8-OH-AAF after rats were treated with luteolin for 24 h prior to AF but luteolin with AF at the same time caused an increase in 1-OH-AAF. In colon tissues, there were significant decreases of AF, 1-OH-AAF, 3-OH-AAF, 5-OH-AAF and 9-OH-AAF after rats were treated with luteolin for 24 h then AF but the amounts of AF, 1-OH-AAF, 5-OH-AAF and 9-OH-AAF decreased and AAF and 8-OH-AAF increased in rats treated with luteolin and AF at the same time. In kidney tissues, there were significant decreases of AF, AAF and 3-OH-AAF after rats were treated with both compounds at the same time, but luteolin for 24 h then AF treatment led to significant decreases of 3-OH-AAF. In liver samples, after rats were treated with luteolin and AF at the same time, the amounts of AAF and 1-OH-AAF significantly decreased but 8-OH-AAF increased. However, rats treated with luteolin for 24 h then with AF led to significant decreases of AAF, 1-OH-AAF and 3-OH-AAF. In feces samples, there were significant increases of AAF, 3-OH-AAF, 7-OH-AAF, 8-OH-AAF and 9-OH-AAF after rats were treated with both compounds at the same time but luteolin for 24 h then AF treatment led to a significant increase of AF, 1-OH-AAF and 8-OH-AAF and a decrease AAF and 3-OH-AAF. In urine samples, there were significant increases of AF, AAF, 1-OH-AAF, 3-OH-AAF, 5-OH-AAF and 9-OH-AAF but a decrease of 8-OH-AAF after rats were treated with both compounds at the same time. However, the luteolin for 24 h then AF treatment led to significant increases of AF, AAF and 1-OH-AAF but

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decreases of 3-OH-AAF and 5-OH-AAF. In cerebrum samples, there were significant increases of AF but decreases of 1-OH-AAF and 8-OH-AAF after rats were treated with both compounds at the same time; luteolin for 24 h then AF treatment of rats led to significant increase of 1-OH-AAF and decreases AF, AAF and 8-OH-AAF. In cerebellum samples, there were significant increases of AAF and decreases of 1-OH-AAF and 3-OH-AAF after rats were treated with both compounds at the same time, there is a significant increase of AAF but decrease of 1-OH-AAF, 3-OH-AAF and 7-OH-AAF after the luteolin treated for 24 h then AF were treated to the rats. In pineal gland samples, there were significant increases of AAF after rats were treated with both compounds at the same time. However, luteolin treated for 24 h then AF were treated to the rats which increase AAF but decrease AF.

Over 70% of cancers are caused by chemical carcinogens (1-3). 2-Aminofluorene (AF) is an arylamine carcinogen which requires host-mediated metabolic activation to initiate carcinogenesis in target tissues. Many reports demonstrated AF to be carcinogenic to organs such as the liver, urinary bladder and other tissues of a variety of animal species (4-6). *N*-acetyltransferase (NAT) plays an important role in AF metabolism (7, 8) and the liver is one of the major organs for AF metabolism (9). In our laboratory, we have indicated that Sprague-Dawley (SD) rats can acetylate AF into acetylfluorene (AAF) which undergoes further metabolism *in vivo* (10, 11).

Luteolin (30,40,5,7-tetrahydroxyflavone), a flavonoid family member, widely occurs in various vegetables, fruits and natural herbal drugs and has several pharmacological properties, including affecting vasodilation (12), anti-inflammatory (13), antioxidant (14) and antiproliferative effects (15), and cancer prevention (16). Recently, it was shown that luteolin can suppress oxidative damage of DNA (17) and down-regulate androgen receptor expression in prostate cancer cells (18). However, there is no information to address whether luteolin affects AF metabolism and distribution *in vivo*. Thus, the present study focused on the effect of oral treatment with luteolin in the distribution and metabolism of AF in an animal model *in vivo*.

Materials and Methods

Chemicals and reagents. Luteolin, 2-aminofluorene (AF), *N*-acetyl-2-aminofluorene (AAF), acetyl carnitine, carnitine acetyltransferase, leupeptin, dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), Tris, bovine serum albumin (BSA) and acetyl-coenzyme A (AcCoA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile, ethyl acetate, potassium phosphates and dimethyl sulfoxide (DMSO) were from Merck Co. (Darmstadt, F.R. Germany). All chemicals used were reagent grade.

Animals and treatment. Eighteen male Sprague-Dawley (SD) rats (160-180 g) were obtained from stock maintained at the animal center of the China Medical University, Taiwan. All rats were

housed in individual cages and were maintained at 25°C on a 12-h light/dark cycle as described (10, 11). Luteolin (30 mg/kg) was administered by syringe in isotonic saline (1 ml of saline administered) (10, 11) and control animals received 1% dimethylsulfoxide (DMSO) only. AF was administered at 50 mg/kg in 1% DMSO to 18 rats. All rats were divided into 3 groups and each group contained 6 rats. Group 1 was treated with AF only (control); group 2 was treated with luteolin and AF at the same time, and group 3 was treated with AF for 24 hours then treated with luteolin (3 mg/kg, dissolved in DMSO).

Animal sample collection for analysis of AF and AF metabolites. All animal were maintained under the same conditions and urine and feces samples were individually collected for 24 h treatment. Animals were sacrificed under CO₂ asphyxiation. Blood samples and bladder, colon, kidney, liver, cerebrum, cerebellum and pineal gland tissues were collected 24 h after treatment. All samples were immediately extracted individually twice with ethyl acetate/methanol (95:5), the solvent evaporated and the residue redissolved in methanol and assayed *via* HPLC (10, 11). An aliquot of the extract from each sample or tissue was injected into a C18 reverse-phase column (Spherisorb 4.6×250 nm) of a Beckman HPLC (pump 168 and detector 126) and eluted at a flow rate of 1.2 ml/min. For all analyses, the solvent system used was 20 mM KH₂PO₄ (pH 4.5)/CH₃CN (53:47) with detection at 280 nm. All compounds were quantified by comparison of the integrated area of the elution peak with that of known amounts of standards (AF, AAF, 1-OH-AAF, 3-OH-AAF, 5-OH-AAF, 7-OH-AAF, 8-OH-AAF and 9-OH-AAF). The total amounts of AF metabolites were calculated as the sum of AAF, 1-OH-AAF, 3-OH-AAF, 5-OH-AAF, 7-OH-AAF, 8-OH-AAF and 9-OH-AAF (10, 11).

Data analysis. Statistical analysis of the data was performed with an unpaired Student's *t*-test.

Results

The analysis data from bladder tissues are shown in Figure 1A. None of the groups exhibited 5-OH-AAF or 7-OH-AAF in their bladder tissue. All groups exhibited AF, AAF, 1-OH-AAF, 3-OH-AAF, 8-OH-AAF and 9-OH-AAF in the examined bladder tissues. The amounts of 1-OH-AAF, 3-OH-AAF, 8-OH-AAF and 9-OH-AAF were significantly different between the control and the concomitant treatment group; 3-OH-AAF and 9-OH-AAF were significantly lower in the group treated with luteolin prior to AF than in the control.

The analysis of the blood is shown in Figure 1B. Again, none of the groups exhibited measurable levels of 3-OH-AAF, 7-OH-AAF or 9-OH-AAF. AF, AAF, 1-OH-AAF, 5-OH-AAF and 8-OH-AAF were found in all groups. The level of 1-OH-AAF was significantly lower in both luteolin-treated groups than in the control group. AF, AAF and 8-OH-AAF were significantly lower in the group treated with luteolin for 24 h before addition of AF than in the control.

The analysis data from colon tissues are shown in Figure 1C. The control (AF only), the luteolin-treated for 24 h before addition of AF and luteolin- and AF-treated at the

same time did not exhibit 7-OH-AAF. All three groups showed various levels of AF, AAF, 1-OH-AAF, 3-OH-AAF, 5-OH-AAF, 8-OH-AAF and 9-OH-AAF in the examined colon tissues. The amounts of AF, AAF, 1-OH-AAF, 5-OH-AAF, 8-OH-AAF and 9-OH-AAF showed a significant difference between the control and the luteolin with AF group treated at the same time. The amounts of AF, 1-OH-AAF, 3-OH-AAF, 5-OH-AAF and 9-OH-AAF showed significant difference between the control and luteolin-treated for 24 h before addition of AF.

The analysis data from kidney tissues are shown in Figure 1D. The control (AF only), the luteolin-treated for 24 h before addition of AF and luteolin- and AF-treated at the same time did not exhibit 5-OH-AAF, 7-OH-AAF and 8-OH-AAF. All three groups showed various levels of AF, AAF, 1-OH-AAF, 3-OH-AAF and 9-OH-AAF in the examined tissues. The amounts of AF, AAF, 3-OH-AAF and 9-OH-AAF showed a significant difference between the control and the luteolin with AF group treated at the same time. The amounts of 3-OH-AAF showed significant difference between the control and luteolin-treated for 24 h before addition of AF.

The analysis data from liver tissues are shown in Figure 1E. The control (AF only), the luteolin-treated for 24 h before addition of AF and luteolin- and AF-treated at the same time did not display 5-OH-AAF, 7-OH-AAF and 9-OH-AAF. All three groups showed various levels of AF, AAF, 1-OH-AAF, 3-OH-AAF and 8-OH-AAF in the examined tissues. The amounts of AAF, 1-OH-AAF and 8-OH-AAF showed a significant difference between the control and the luteolin with AF group treated at the same time. The amounts of AAF, 1-OH-AAF and 3-OH-AAF showed significant difference between the control and luteolin-treated for 24 h before addition of AF.

The analysis data from feces are shown in Figure 1F. The control (AF only), the luteolin-treated for 24 h before addition of AF and luteolin- and AF- treated at the same time exhibited various levels of AF, AAF, 1-OH-AAF, 3-OH-AAF, 5-OH-AAF, 7-OH-AAF, 8-OH-AAF and 9-OH-AAF. The amounts of AAF, 3-OH-AAF, 7-OH-AAF, 8-OH-AAF and 9-OH-AAF showed a significant difference between the control and the luteolin with AF group treated at the same time. The amounts of AF, AAF, 1-OH-AAF, 3-OH-AAF and 8-OH-AAF showed a significant difference between the control and luteolin-treated for 24 h before addition of AF.

The analysis data from urine are shown in Figure 1G. The control (AF only), the luteolin-treated for 24 h before addition of AF and luteolin- and AF- treated at the same time displayed various levels of AF, AAF, 1-OH-AAF, 3-OH-AAF, 5-OH-AAF, 7-OH-AAF, 8-OH-AAF and 9-OH-AAF. The amounts of AF, AAF, 1-OH-AAF, 3-OH-AAF, 5-OH-AAF, 8-OH-AAF and 9-OH-AAF showed significant difference between the control and the luteolin with AF group treated at the same time. The amounts of AF, AAF,

1-OH-AAF, 3-OH-AAF and 5-OH-AAF showed significant difference between the control and luteolin-treated for 24 h before addition of AF.

The analysis data from cerebrum are shown in Figure 1H. The control (AF only), the luteolin-treated for 24 h before addition of AF and luteolin- and AF-treated at the same time exhibited various levels of AF, AAF, 1-OH-AAF, 3-OH-AAF and 8-OH-AAF. The amounts of AF, 1-OH-AAF and 8-OH-AAF showed a significant difference between the control and the luteolin with AF group treated at the same time. The amounts of AF, AAF, 1-OH-AAF and 8-OH-AAF showed a significant difference between the control and luteolin-treated for 24 h before addition of AF.

The analysis data from cerebellum are shown in Figure 1I. The control (AF only), the luteolin-treated for 24 h before addition of AF and luteolin- and AF-treated at the same time displayed various levels of AF, AAF, 1-OH-AAF, 3-OH-AAF and 7-OH-AAF. The amounts of AAF, 1-OH-AAF and 3-OH-AAF showed significant difference between the control and the luteolin with AF group treated at the same time. The amounts of AAF, 1-OH-AAF, 3-OH-AAF and 7-OH-AAF showed significant difference between the control and luteolin-treated for 24 h before addition of AF.

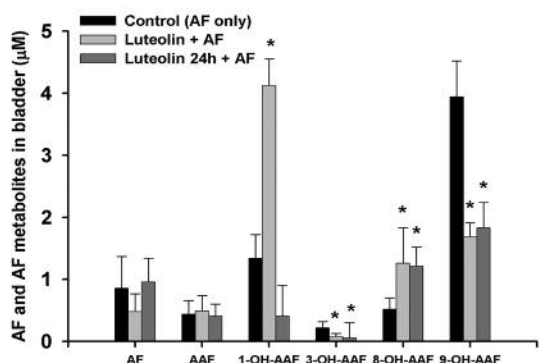
The analysis data from pineal gland tissues shown in Figure 1J. The control (AF only), the luteolin-treated for 24 h before addition of AF and luteolin- and AF-treated at the same time displayed various levels of AF and AAF. The amounts of AAF showed a significant difference between the control and the luteolin with AF group treated at the same time. The amounts of AF and AAF showed a significant difference between the control and luteolin-treated for 24 h before addition of AF.

Discussion

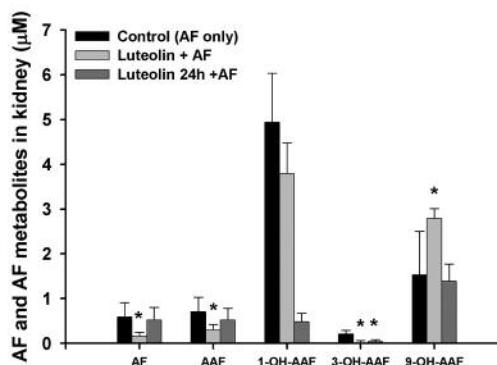
The purpose of this study was a) to investigate the distribution and metabolites of AF; and b) to further investigate the effect of luteolin on the *in vivo* AF metabolism in an animal model. Although metabolism and mutagenesis studies of AF and AAF have been carried out with animal microsomal, S9, and intact hepatocyte preparation (19-21), the effect of luteolin on the metabolism and distribution of AF *in vivo* has not been reported.

NAT plays an important role in AF-induced malignancy in mammals and AF acetylated by NAT is present in many tissues of experimental animals and humans, and is involved in chemical carcinogenesis (22, 23). Increased levels of NAT activity are associated with increased sensitivity to the mutagenic effects of arylamine carcinogens (24) but reduced NAT activity of the liver is associated with several disease processes (breast and bladder cancer) (8, 23). Our previous studies had shown that luteolin affected NAT activity and gene expression in many human cancer cell lines. However,

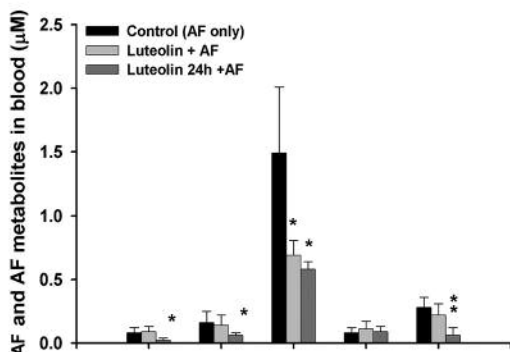
A. Bladder



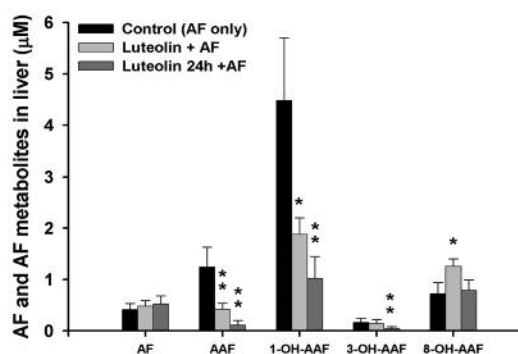
D. Kidney



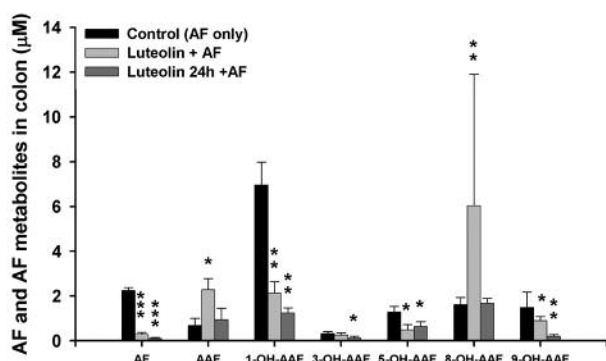
B. Blood



E. Liver



C. Colon



F. Feces

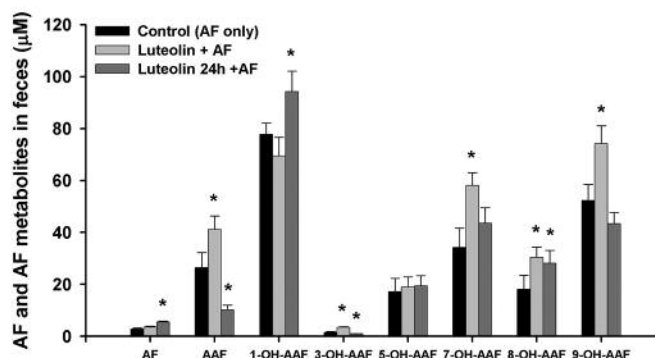


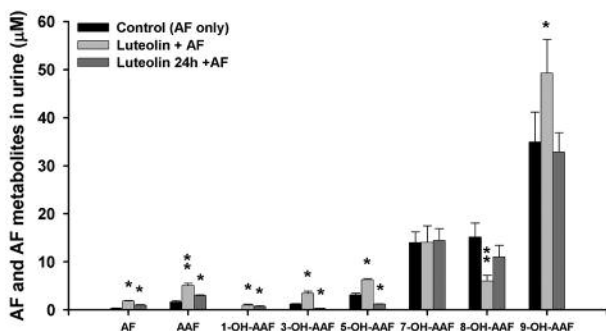
Figure 1. continued

there is no available information about the effect of dietary luteolin on distribution and metabolism of AF *in vivo* in rat tissues after oral treatment with AF. Therefore, the present studies were focused on the effects of dietary luteolin on AF distribution and metabolism in bladder, blood, brain, colon, kidney, liver, feces and urine samples from SD rats. The results from this study demonstrated that luteolin could affect

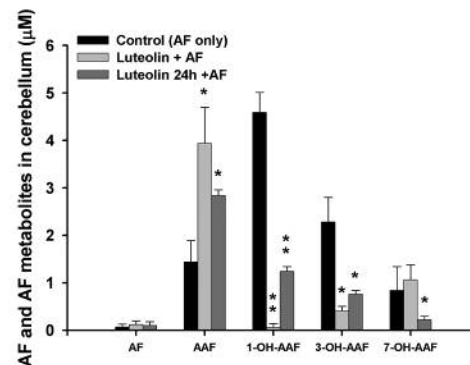
N-acetylation and the metabolism of AF *in vivo* in the examined rat tissues, including brain tissues.

Luteolin, a dietary flavonoid, was demonstrated to exert chemopreventive and anticarcinogenic effects against 1, 2-dimethyl hydrazine (DMH)-induced colon cancer (25) and it also acted as a potent chemopreventive agent against colon carcinogenesis (26). The reason for selecting pretreatment with

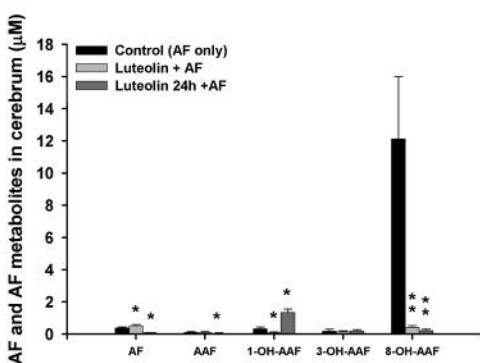
G. Urine



I. Cerebellum



H. Cerebrum



J. Pineal gland

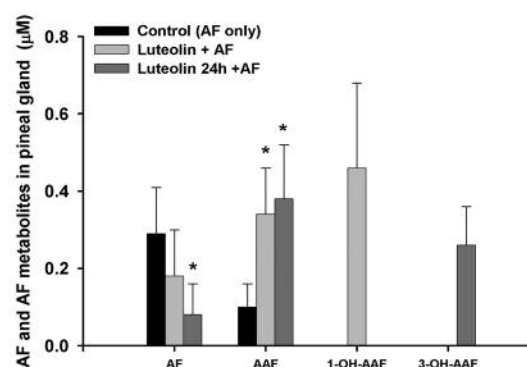


Figure 1. Distribution of AF and AF metabolites in various tissues from male SD rats after oral treatment with AF: bladder (A), blood (B), colon (C), kidney (D), liver (E), feces (F), urine (G), cerebrum (H), cerebellum (I) and pineal gland (J). AF and AF metabolites were analyzed by HPLC as described. Values are mean±SD n=6; *p<0.05, **p<0.01, ***p<0.01.

luteolin for 24 h before the addition of AF was based on our earlier findings that a day of oral treatment with ellagic acid (11) and berberine (27) significantly affected AF metabolism and distribution in SD rats. One day of administration may suffice to induce large changes in liver enzyme activity. Our previous studies also showed that berberine (27) and ellagic acid (28) affected the *N*-acetylation of AF *in vivo* in the examined cerebrum, cerebellum and pineal gland of rats. The present studies also showed that luteolin affected the total amounts of AF and AF metabolites in all the examined tissues (bladder, blood, colon, kidney, liver feces and urine) including brain tissues (cerebrum, cerebellum and pineal gland).

There are many reports on agents which affect *N*-acetylation of drugs. In the rat (21) and the rabbit (22), Freund's adjuvant can stimulate the reticuloendothelial system leading to an increase rate of *N*-acetylation *in vivo*. Again in the rat, pretreatment with tilorone resulted in an increased rate of *N*-acetylation of procainamide (29) and AF (10) *in vivo*, while in the rabbit, chronic administration of hydrocortisone led to enhanced acetylation of sulfamethazine (23). The

decrease of *N*-acetylation and total amounts of AF metabolites in the present study may suggest that luteolin may inhibit the oxidative metabolism of AF, since the elimination pathways of procainamide include renal excretion of unchanged drug, acetylation and oxidative metabolism (30).

The present data also showed that luteolin affected AF metabolism and distribution *in vivo*. Small peaks in the profile of HPLC were also found but could not be isolated, purified or identified because of their small quantities. Therefore, the nature of the metabolites corresponding to unknown peaks in all the examined samples remains unknown.

In conclusion, when AF was given to SD rats orally with or without luteolin pretreatment or at the same time with AF, the 24 h samples extracted and analyzed by HPLC indicated that luteolin reduced the rate of AF *N*-acetylation in bladder, blood, colon, kidney and liver tissues. However, the results also showed that luteolin affected the distribution and metabolites in brain tissues (cerebrum, cerebellum and pineal gland). Therefore, we suggest that predisposition to carcinogenicity may be influenced by specific chemopreventive agents.

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