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Green tea: A promising anticancer agent for renal cell carcinoma

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ABSTRACT

Renal cell carcinoma (RCC) is one of the most lethal amongst the urologic malignancies, comprising three percent of all human neoplasms, and its incidence appears to be rising. RCC is refractory to both chemotherapy and radiotherapy. Therefore, the discovery of new strategies for therapeutic intervention remains a priority. Green tea (*Camellia sinensis*) and tea polyphenols have been proposed to exert protective effects against several types of cancer, based on preclinical and clinical trial data; however, the anticarcinogenic activity of green tea towards RCC is unknown. In this study, a targeted metabolite analysis on a green tea leaves methanolic extract was performed by HPLC/DAD and the antiproliferative activity of the extract was assayed using human renal cancer cell lines A-498 and 769-P. The total phenolic content was very high (31.8% of methanolic extract), and the main compounds were flavan-3-ols (94.3% of the total phenolic content), and especially (−)-epigallocatechin-3-gallate (35.9% of the total phenolic content). In addition, two methylxanthines – theophylline and caffeine – were also present in the extract, caffeine being the most abundant. Green tea extract strongly inhibited the growth of both RCC cell lines in a concentration-dependent manner, with IC_{50} values of 54 ± 10 and 129 ± 28 μg/ml for A-498 and 769-P cells, respectively. This is the first report showing that green tea is likely to be an effective anticancer agent for renal cell carcinoma.

1. Introduction

Kidney cancer presently ranks 7th in the leading causes of cancer amongst men and 8th amongst women in the United States (Jemal et al., 2009). Although it only accounts for about 3% of all solid neoplasms, the incidence of kidney malignancies has increased over the past three decades (Mathew, Devesa, Fraumeni, & Chow, 2002). It is estimated, by the American Cancer Society, that over 57,760 new cases of kidney cancer will be diagnosed and 12,980 deaths from this disease will occur in the current year (2009) (Jemal et al., 2009). Renal cell carcinomas (RCC) arise from the proximal tubular epithelium and account for the majority of renal tumour malignancies (Amato, 2000). Symptoms arise late in the course of disease; hence, approximately 50% of patients present with locally advanced or metastatic disease and have poor prognosis due to the refractory nature of RCC to conventional chemotherapy and radiotherapy regimens (Motzer et al., 2000). Therefore, the discovery of new strategies for therapeutic intervention remains a priority.

Green tea, the infusion prepared from dried leaves of *Camellia sinensis* (L.) O. Kuntze (Theaceae family), is a major beverage and consumed worldwide. It has been considered a health-promoting beverage since ancient times. Green tea contains a mixture of over 2000 different phytochemicals, such as phenolic compounds, methylxanthines, carbohydrates, proteins, free amino acids, L-ascorbic acid and other organic acids, volatile compounds, lipids, carotenoids, chlorophylls, minerals and trace elements (Moderno, Carvalho, & Silva, 2009). Phenolic compounds constitute the major active group of tea leaves' components, due simultaneously to their higher relative abundance (up to 30% of the dry weight of the water-extractable material) and bioactive properties (Moderno et al., 2009; Yang et al., 2006). In fact, the beneficial effects of green tea polyphenols, particularly of (−)-epigallocatechin-3-gallate (EGCG), in the prevention of cancer have been recognised in many animal models of carcinogenesis and human cancer cell lines (Ju, Lu, Lambert, & Yang, 2007; Yang & Chung, 1999; Yang, Maliakal, & Meng, 2002). However, the molecular mechanisms for these anticancer actions are not fully understood. They are most likely related to the biochemical activity of the tea polyphenols, including their antioxidant effect, protection of DNA from

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damage and/or methylation, inhibition of proteasome activity in tumour cells, induction of apoptosis in tumour or transformed cells, cell cycle regulation, and inhibition of cell proliferation and tumour-promotion related events (Carlson, Bauer, Vincent, Limburg, & Wilson, 2007; Chen & Dou, 2008; Yang, Wang, Lu, & Picinich, 2009).

For the past two decades, much research has been developed in order to discover natural compounds with potential benefits in cancer prevention and treatment. Major limitations of the anticarcinogenic agents presently under study in human cancer investigation trials are their high cost, the inevitability of either intravenous or subcutaneous administration on a long-term basis and often their tendency to destroy some healthy cells, along with cancerous cells. Thus, the potential of green tea or its polyphenols as candidates for chemoprevention and/or chemotherapy is high because of its low cost, wide availability, and apparent low toxicity. Furthermore, studies comparing the *in vitro* responses of normal and malignant cells to tea polyphenols have revealed that immortalised cells were more sensitive (in terms of growth inhibition and apoptosis induction) than were normal cells (Ahmad, Gupta, & Mukhtar, 2000; Chen, Schell, Ho, & Chen, 1998).

Although anticancer properties of green tea have been shown in several cancer types, its effect in RCC is not yet known. Therefore, the present study was designed to evaluate the antiproliferative potential of green tea extract in human RCC-derived cell lines. As far as we know, this is the first time that kidney anticancer activity of green tea extract is evaluated.

2. Materials and methods

2.1. Reagents

Methanol was purchased from Merck (Darmstadt, Germany). All other reagents and standards were obtained from Sigma (St. Louis, USA).

2.2. Plant material and extraction

A *C. sinensis* dried leaves sample was purchased on the Portuguese market. Dried plant material (1.7 g) was thoroughly mixed with methanol (3 × 25 ml). The extract was filtered and concentrated to dryness under reduced pressure (0.3 g) and then redissolved in 6 ml of dimethylsulfoxide (DMSO) to obtain a 50 mg of extract/ml stock solution.

2.3. Identification and quantification of green tea polyphenols and methylxanthines

Stock solution (20 μl) was analysed using an analytical HPLC unit (Gilson) and a C18 Spherisorb ODS2 column (25.0 × 0.46 cm; 5 μm, particle size) from Waters (Ireland). The solvent system used was a gradient of water–formic acid (19:1) (A) and methanol (B), starting with 5% methanol and installing a gradient to obtain 15% B at 3 min, 25% B at 13 min, 30% B at 25 min, 35% B at 35 min, 45% B at 39 min, 45% B at 42 min, 50% B at 44 min, 55% B at 47 min, 70% B at 50 min, 75% B at 56 min and 80% B at 60 min, at a solvent flow rate of 0.9 ml/min (Costa et al., 2009). Detection was achieved with a Gilson diode array detector (DAD). Spectral data from all peaks were accumulated in the range 200–400 nm, and chromatograms were recorded at 280, 320 and 350 nm. Chromatographic data were processed by Unipoint® System software from Gilson Medical Electronics (Villiers le Bel, France). The compounds in each sample were identified by comparing their retention times and UV–vis spectra in the 200–400 nm range with the library of spectra previously compiled by the authors.

Quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. 3-*O*- and 4-*O*-caffeoylquinic and 3,5-*O*-dicaffeoylquinic acids were quantified as 5-*O*-caffeoylquinic acid. The other compounds were quantified as themselves.

2.4. Cell culture materials

Human renal epithelial cancer cells A-498 (ATCC HTB-44) and 769-P (ATCC CRL-1933) were cultured in RPMI 1640 medium (GlutaMax Media, Gibco BRL Life Technologies) with 25 mM HEPES containing 10% heat-inactivated fetal bovine serum (Gibco, Invitrogen), 100 U/ml penicillin (Gibco, Invitrogen), and 100 μg/ml of streptomycin (Gibco, Invitrogen). For the assay, cells were grown in T-75 culture flasks in a humidified incubator at 37 °C under 95% air and 5% CO_2 mixture.

2.5. Cell proliferation assay

Cell proliferation was measured using the mitochondrial MTT assay, as previously described (Carvalho, Hawksworth, Milhazes, Borges, & Monks, 2002). MTT is a yellow water-soluble dye that is reduced in viable cells to an insoluble purple-coloured product, MTT-formazan (3-[4,5-dimethylthiazol-2-yl]-3,5-diphenylformazan) – by the mitochondrial enzyme succinate dehydrogenase. A-498 and 769-P cells were subcultured at 5×10^3 cells per well of a 96-well microplate system and incubated for 24 h to allow cell attachment. Cells were then treated with different concentrations of green tea methanolic extract. Green tea stock solution (50 mg extract/ml in DMSO) was accurately diluted in medium to obtain final concentrations of 31.3, 62.5, 125, 250, and 500 μg extract/ml. By these serial dilutions, the DMSO final concentration was never higher than 1% and the same concentration was used for solvent-control wells. The plates were incubated for 48 h under the same conditions. At the end of exposure time, the medium was removed and the cells were washed once with fresh culture medium. Subsequently the cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 4 h with 200 μl of 0.5 mg/ml of MTT (final concentration). MTT solution was then removed and intracellular MTT-formazan was solubilised in 100 μl of dimethyl sulfoxide and the absorbance measured at 550 nm in a microplate reader. Cell viability was measured as the percentage of absorbance compared to control. The 50% inhibitory concentration (IC_{50}) values, defined as the amount of extract that inhibits 50% of cell growth, were calculated from concentration–response curves, following a 48 h exposure time. Three independent experiments, performed in triplicate, were used for these calculations.

2.6. Statistical analysis

Statistic analysis was performed using the Statistical Package for Social Sciences (SPSS, version 16.0) for Windows. Multiple comparisons were performed by one-way ANOVA supplemented with Tukey's HSD post hoc test. Significance was accepted at P lower than 0.05.

3. Results

3.1. Phenolic and methylxanthine profile of green tea extract

The methanolic extract of green tea leaves is an especially rich source of phenolic compounds (318 g/kg). Its phenolic profile is composed of 15 constituents (Fig. 1 and Table 1): an hydroxybenzoic acid – gallic acid, four hydroxycinnamic acid derivatives – 3-*O*-, 4-*O*- and 5-*O*-caffeoylquinic acids and 3,5-*O*-dicaffeoylquinic

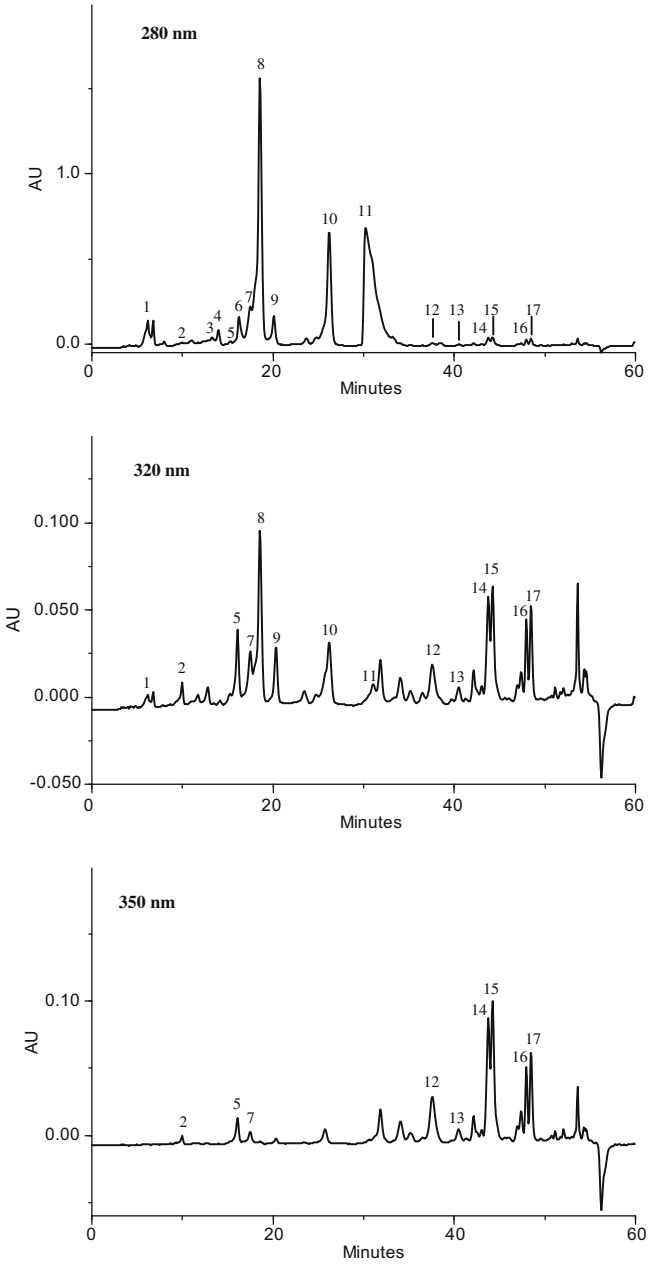

Fig. 1. HPLC profile of green tea methanolic extract. (1) gallic acid, (2) 3-*O*-caffeoylquinic acid, (3) catechin, (4) epigallocatechin, (5) 4-*O*-caffeoylquinic acid, (6) theophylline, (7) 5-*O*-caffeoylquinic acid, (8) epigallocatechin-3-gallate, (9) epicatechin, (10) epicatechin-3-gallate, (11) caffeine, (12) myricetin-3-glucoside, (13) 3,5-*O*-dicaffeoylquinic acid, (14) quercetin-3-galactoside, (15) quercetin-3-rutinoside, (16) kaempferol-3-glucoside, (17) kaempferol-3-rutinoside.

acid, five flavan-3-ols (catechins) – catechin, epigallocatechin, epigallocatechin-3-gallate, epicatechin, epicatechin-3-gallate, and five flavonol glycosides – myricetin-3-glucoside, quercetin-3-galactoside, quercetin-3-rutinoside, kaempferol-3-glucoside and kaempferol-3-rutinoside. As expected, the most representative class of phenolic compounds is the catechin family (94.3% of the total phenolic content) and the most abundant is epigallocatechin-3-gallate (114 g/kg, representing 38.1% of the total catechin in tea).

Many other authors have already identified and quantified green tea polyphenols (Baptista, Tavares, & Carvalho, 1998; Marques & Farah, 2009; Seeram et al., 2006; Stewart, Mullen, & Crozier, 2005). In all of these works, catechins were considered the major bioactive constituents (representing about 30% of the

Table 1
Phenolic and methylxanthine composition of green tea methanolic extract. Values are expressed as means ± standard deviation of three assays for each sample (g/kg of methanolic extract).

Phenolic compound/methylxanthine	Content (g/kg of extract)
Gallic acid	3.45 ± 0.02
3-*O*-Caffeoylquinic acid	0.34 ± 0.01
Catechin	9.60 ± 0.74
Epigallocatechin	110 ± 6.70
4-*O*-Caffeoylquinic acid	1.04 ± 0.15
Theophylline	6.61 ± 0.35
5-*O*-Caffeoylquinic acid	0.81 ± 0.02
Epigallocatechin-3-gallate	114 ± 3.22
Epicatechin	28.6 ± 1.42
Epicatechin-3-gallate	37.8 ± 2.19
Caffeine	53.9 ± 3.85
Myricetin-3-glucoside	1.40 ± 0.05
3,5-*O*-Dicaffeoylquinic acid	0.31 ± 0.00
Quercetin-3-galactoside	2.88 ± 0.04
Quercetin-3-rutinoside	5.17 ± 0.20
Kaempferol-3-glucoside	0.84 ± 0.04
Kaempferol-3-rutinoside	1.86 ± 0.11

dry weight of the water-extractable material) and, amongst them, epigallocatechin-3-gallate is the most abundant. Some researchers also reported the presence of phenolic acids and/or flavonols and their derivatives in green tea extracts (Baptista et al., 1998; Marques & Farah, 2009; Stewart et al., 2005) but, as far as we know, this is the first time that 3,5-*O*-dicaffeoylquinic acid is quantified.

In this extract, two methylxanthines – theophylline and caffeine – were also found (Fig. 1 and Table 1), caffeine being the most abundant (5.39%). Our study is in accordance with that of Yang et al. (2006), who reported that, besides catechins, caffeine is major active constituent of green tea, representing between 3% and 6% of the dry weight of the water-extractable material.

3.2. Inhibition of human renal cancer cell growth by green tea

Green tea extract was evaluated for its ability to inhibit the growth of A-498- and 769-P RCC-derived cell lines. Fig. 2 presents the concentration effectiveness of green tea extract on viability of A-498 and 769-P cells. Five different concentrations of extract (31.3, 62.5, 125, 250, and 500 μg of extract/ml) were applied. Green tea remarkably inhibited the growth of both renal cancer cell lines in a concentration-dependent manner, although there was no significant effect for the lowest concentration of extract (31.3 μg/ml) against 769-P cells. The inhibitory effect of green tea extract ranged from 29% to 97% for the A-498 cells and 26% to 89% for the 769-P cells. The IC_{50} values obtained for antiproliferative activity of green tea extract were of 54 ± 10 and 129 ± 28 μg/ml, for A-498 and 769-P cells, respectively, at 48 h.

4. Discussion

The potential cancer preventive and therapeutic properties of green tea and tea polyphenols have been studied extensively in recent years. Inhibition of carcinogenesis by green tea polyphenols has been demonstrated in many different animal models, including those for cancer of the skin, lung, digestive tract (oral cavity, esophagus, stomach, small intestine, colon), bladder, liver, pancreas, prostate and mammary glands (reviewed in Ju et al., 2007; Yang et al., 2002). Tea polyphenolic compounds have also been demonstrated to inhibit the growth of a variety of cancer cell lines (Yang & Chung, 1999). Most of these anticancer actions of green tea, found in *in vivo* and *in vitro* studies, are believed to be mediated by tea polyphenols, and especially by the major component EGCG. In fact, EGCG exhibited strong antiproliferative and

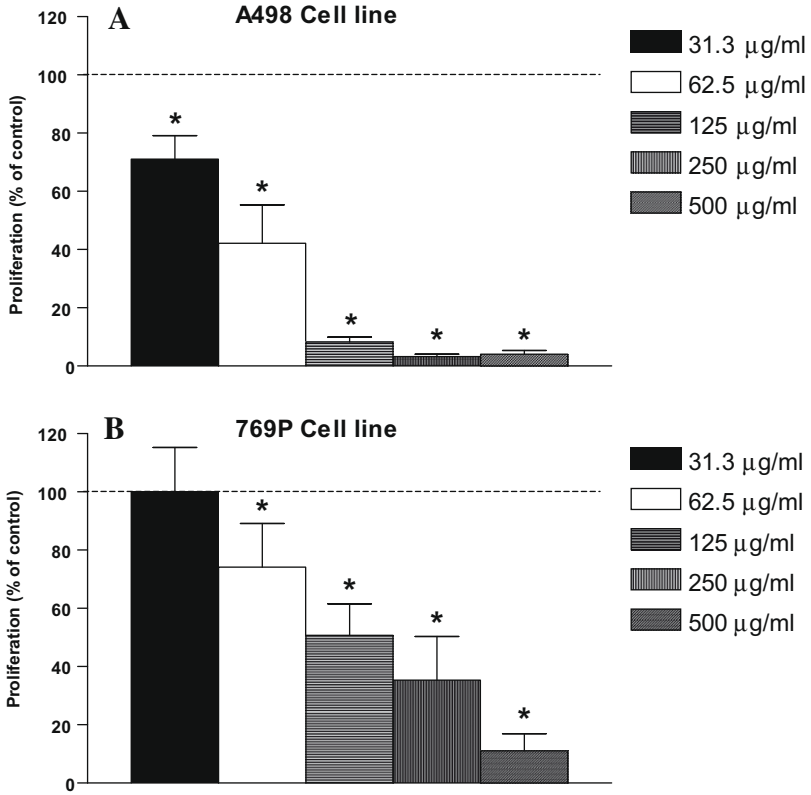

Fig. 2. Concentration effectiveness of green tea extract on growth inhibition of (A) A-498 and (B) 769-P cells. Five concentrations of extracts (31.3, 62.5, 125, 250, and 500 μg/ml) were applied. Values are presented as means ± SD of three independent experiments, performed in triplicate. * $P < 0.05$, as compared with control.

anticancer effects against lung (Sadava, Whitlock, & Kane, 2007; Yang, Liao, Yang, & Lu, 2005), skin (Ravindranath, Ramasamy, Moon, Ruiz, & Muthugounder, 2009), bladder (Qin et al., 2007), cervical (Ahn, Huh, & Bae, 2003a, 2003b), prostate (Albrecht, Clubbs, Ferruzzi, & Bomser, 2008), pancreatic (Shankar, Ganapathy, Hingorani, & Srivastava, 2008), breast (Thangapazham et al., 2007) and hepatic (Shirakami et al., 2009) cancer cells.

Green tea chemical composition is well-known. Its major polyphenolic compounds (known as catechins) are EGCG, (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and (−)-epicatechin (EC). Catechin, gallocatechin, epigallocatechin digallates, epicatechin digallate and 3-*O*-methyl gallocatechin gallate are also present but in smaller quantities (reviewed in Moderno et al., 2009; Yang, Lambert, Ju, Lu, & Sang, 2007). Recent mechanistic studies evidenced that tea polyphenols, in particular EGCG, afford protection against cancer through multiple biological mechanisms. Green tea polyphenols are recognised for their strong antioxidant properties (reviewed in Moderno et al., 2009; Yang et al., 2002), and these properties may play a key role in the anticancer activities (Rietveld & Wiseman, 2003; Shankar, Ganapathy, & Srivastava, 2007). Tea polyphenols scavenge harmful reactive nitrogen and oxygen species, such as superoxide radical, singlet oxygen, hydroxyl radical, peroxyl radical, nitric oxide, nitrogen dioxide, and peroxynitrite (Costa et al., 2009; Rietveld & Wiseman, 2003). They are also strong metal chelators, inactivating redox-active transition metal ions that would otherwise catalyse free radical formation. One hypothesis explaining anticarcinogenic effects is that tea polyphenols can protect pre-malignant cells from oxidative breakage of cellular DNA. However, it has been suggested that the antioxidant properties of these phenolic compounds may not entirely explain their chemopreventive effects. Other mechanisms include inhibition of angiogenesis and metastasis, and induction of growth arrest and apoptosis through regulation of multiple signalling pathways (Carlson et al., 2007; Fassina et al., 2004; Yang et al., 2009). In detail, EGCG regulates expression of VEGF, matrix metalloproteinases (MMPs), uPA, IGF-1, EGFR, cell cycle regulatory proteins and inhibits NF-kB, PI3-K/Akt, Ras/Raf/MAPK and AP-1 signalling pathways, thereby causing strong cancer chemopreventive effects (Shankar et al., 2007, 2008; Yamakawa et al., 2004).

Inhibition of cell growth and the induction of apoptosis in different cancer cell lines, including those from skin, lung, stomach, prostate and blood, by EGCG and other tea polyphenols has been reported and reviewed (Yang & Chung, 1999; Yang, Liao, Kim, Yurkow, & Yang, 1998b, 2002). However, studies investigating the anticarcinogenic effect of green tea extract or of tea catechins in RCC cells are scarce. Gu, Ding, Xia, and Fang (2009) have recently shown that EGCG inhibits the proliferation and induces apoptosis in the human renal carcinoma cell line 786-0 through tissue factor pathway inhibitor-2 (TFPI-2) overexpression. In another study, Roomi, Ivanov, Kalinovsky, Niedzwiecki, and Rath (2006) examined the anticancer effects of a specific nutrient mixture containing lysine, proline, arginine, ascorbic acid and green tea extract using the same renal adenocarcinoma cell line (786-0). Although this combination showed no significant effect on renal cancer cell growth, it strongly inhibited the secretion of matrix metalloproteinases MMP-2 and MMP-9. MMPs are a family of zinc-dependent neutral endopeptidases that elicit degradation of the extracellular matrix and basement membranes, ultimately leading to tumoral progression (Katiyar, 2006). Our study shows, for the first time, that green tea extract markedly prevented the proliferation of human RCC cells, thus contributing to the developing body of data which demonstrates that green tea extract presents effective human cancer cell antiproliferative activities *in vitro*. Most recent mechanistic studies concentrate mainly on the beneficial properties of the most abundant polyphenol in green tea, i.e. EGCG. Nevertheless, in view of the complexity of the mechanisms proposed for the chemopre-

ventive properties of tea polyphenols, it is likely that anticarcinogenic effects attributed to green tea may be based on synergistic, additive or antagonistic interactions of polyphenols present in this medicinal plant. Corroborating this, Thangapazham et al. (2007) demonstrated that the green tea polyphenol mixture was needed at a lower concentration when compared to EGCG to induce similar antiproliferative effects in a human breast cancer MDA-MB-231 cell line. Nonetheless, there are other compounds, present in green tea, of great interest for human health, such as methylxanthines, namely caffeine and theophylline, L-theanine (a free amino acid which is considered characteristic of tea), L-ascorbic acid, fluoride, chromium and manganese. For example, some studies have reported a key role for caffeine in the inhibitory activity of skin and lung tumorigenesis in rats by tea (Chung et al., 1998; Huang et al., 1997). Thus, combinations of constituents present in plant extract, rather than the effect of a specific phytochemical or a class of phytochemicals, may be highly decisive for its final biological activity.

Although the effective concentrations of green tea extract required for inhibition of RCC proliferation in this study were very low and in the same range of concentrations as other *in vitro* studies, they are not physiologically relevant. In fact, maximum plasmatic levels of tea catechins after oral administration of green tea extract (1.5–4.5 g) to healthy volunteers was found to be 0.326 μg/ml for EGCG, 0.550 μg/ml for EGC, and 0.190 μg/ml for EC (Yang et al., 1998a). However, pharmacokinetics studies have demonstrated that EGC and EC (but not EGCG) are excreted in the urine (Chen, Lee, Li, & Yang, 1997). Of note, in our extract, is that EGC is present in concentrations similar to EGCG. Therefore, the kidney may actually be exposed to high levels of effective tea polyphenols after ingestion. Further studies are clearly necessary to clarify the clinical potential of green tea for therapy applications in RCC patients.

5. Conclusion

This is the first report showing that green tea is a promising anticancer agent for renal cell carcinoma. These antitumoral properties are most likely related to the high content of polyphenolic compounds in green tea. Notably, renal cell carcinoma is highly resistant to current chemotherapeutic regimens and studies conducted on cell cultures have revealed it to be a slightly responsive cancer cell line with a few natural extracts showing significant growth inhibition effects toward these cells (Boivin et al., 2009). Additional studies in animal models and clinical trials are required in order to fully evaluate the potential of this medicinal plant in the prevention and/or treatment of kidney cancer.

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