Abstract. The flavonoid naringin is a polyphenolic compound that naturally occurs in citrus. Patients with cancer generally present features of malnutrition and cachexia. Levels of the proinflammatory cytokines tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) are raised in patients with cancer. This study was designed to analyze the in vivo effect of naringin in the therapeutic treatment of rats bearing Walker 256 carcinosarcoma (W256). Rats were treated intraperitoneally with different doses of naringin (10, 25 and 35 mg/kg), for 50 days. At 25 mg/kg, naringin inhibited tumor growth by ~75%. With this treatment, TNF-α and IL-6 levels decreased (p<0.05) in comparison with the control. In addition, two rats presented complete tumor regression. Inhibition of tumor growth, survival increase and the reduction of TNF-α and IL-6 levels in rats bearing W256 treated with naringin strongly suggest that this compound has potential as an anticarcinogenic drug.

Cancer has been established as a public health problem worldwide. According to a report by the International Agency for Research on Cancer (IARC)/WHO (1), the overall impact of cancer more than doubled in 30 years, and is now the second leading cause of death by disease in developed countries, behind cardiovascular disease. One of the side-effects of cancer is cachexia, a state characterized by several metabolic disorders, anorexia and involuntary weight loss, resulting in decreased quality of life (2). More than half of all cancer patients suffer from this syndrome. Cachexia occurs in more than 80% of patients with advanced cancer (2), being responsible for a decline of around 60% of body mass in relation to ideal weight (3) and the main cause of death in more than 20% of patients (4). Antineoplastic treatment is not well tolerated by patients with cachexia, who are more sensitive to the side-effects (5). Complications associated with worsening cachexia are closely linked with the balance between pro-cachectic and anti-cachectic factors (6). Among them, there are the pro-inflammatory cytokines responsible for induction of hepatic acute phase proteins (7). These cytokines contribute to immunosuppression and tumor cell proliferation, tissue remodeling and angiogenesis (8). Different experimental protocols have shown that cytokines have effects on appetite and are potent inducers of weight loss. Interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) stand out among these cytokines (9). TNF-α receptors are found in the hypothalamic area and regulate food intake (10). Administration of increasing doses of TNF-α induces cachexia in animals, resulting from reduced food intake and negative nitrogen balance (11).

For a long time, fields of science related to human health have concentrated efforts on substances that have a high medical potential. Natural products which are regularly eaten in large quantities with components of human diets with low or negligible toxicity are gaining prominence (12). The search for new anticancer compounds in medicinal plants and traditional foods is a promising and a realistic strategy for cancer prevention (13). Naringin, a flavanone glycoside, is the most abundant flavonoid in orange and can be found in other species of citrus fruits, such as grapefruit and Kino (14). Many biological functions have been described for naringin, including anti-inflammatory, antioxidant (15), antimicrobial (16), antiviral (17), antiulcer (18) and anticancer, as well as inhibiting cell proliferation in several human cancer cell lines, including those of the stomach,
Materials and Methods

Materials. Naringin was obtained from Sigma Chemical Co. (St. Louis, MO, USA). MILLIPLEX Map Rat cytokine IL-6 and TNF-α were obtained from MILLIPORE (Cat. No. RCYTO-80K; Billerica, Massachusetts, United States). All the other reagents used were of analytical grade.

Animals. Male Wistar rats (9 weeks-old) were obtained from the Centro de Bioterismo da UNICAMP (Campinas, São Paulo, Brazil). The animals were housed in stainless steel wire-bottomed cages and acclimatized under laboratory conditions (18-24˚C, 60% humidity, 12h light/dark cycles, with free access to diet and water). The general United Kingdom Coordinating Committee on Cancer Research (UKCCCR) guidelines (27) for animal welfare were followed, and the protocols were approved by the institutional Committee for Ethics in Animal Research (CEEA/IB/UNICAMP No. 1088-1).

The rats assigned to the tumor-bearing groups were inoculated in the right flank with a subcutaneous injection of W256 cells (approx. 0.25x10⁶ viable cells in 0.5 ml of saline solution) obtained from an ascitic tumor-bearing rat, as described by Gomes-Marcondes et al. (28). Naringin was dissolved in 0.3 ml of 0.9% physiologic salt solution and 20% propylene glycol and i.p. administered for five consecutive days per week.

Survival and naringin concentration that inhibits 50% tumor growth (ED₅₀). All the animals received W256 cells. The naringin treatment started 3h before the rat tumor cell inoculation. The animals were randomly assigned to four different groups (10 animals each) for dose response (ED₅₀) and survival determinations: tumor group (T) and groups that received naringin at different doses – 10, 25 and 35 mg/kg (TN10, TN25 and TN35 respectively). The rats were treated and monitored until death. Tumor weight was determined three times a week (Mondays, Wednesdays and Fridays). The tumor weights were calculated from the formula: weight=0.79768+(0.000456(length×width×thickness of the tumor)), according to Gomes-Marcondes (29). The weight gain in 21 days was determined through the formula: weight gain=(Fw-Tw)–Iw; where, Fw: rat final weight, in the sacrifice day; Tw: tumor weight and Iw: rat initial weight, before tumor induction.

Therapeutic treatment. Naringin 25mg/kg (determined by ED50) was administered for 21 consecutive days. The animals were randomly assigned to four different groups (10 animals each): control group (C), tumor group (T), naringin group (N25), and rats bearing tumor treated with naringin (TN25). The animals were sacrificed by cervical dislocation 24h after the last dose and livers and primary tumor were collected, washed with saline, frozen in liquid nitrogen and stored in a biofreezer (−80˚C) for subsequent analyses.

IL-6 and TNF-α assays. The assays for IL-6 and TNF-α detection were performed in liver and tumor tissues with MILLIPLEX Map Rat cytokine IL-6 and TNF-α kit (Cat. No. RCYTO-80K) according to the manufacturer’s instructions. After 21 days the animals were sacrificed by cervical dislocation. Liver and tumor were collected, washed with saline solution, homogenized in 1M acetate buffer, pH 5 (1:4), centrifuged at 10.000 rpm for 40 min and the supernatant was used for the measurements.

Statistical analysis. The survival curves were derived by the Kaplan–Meier method (30) and the statistical comparisons among the groups were carried out using the Mantel–Haenszel log-rank test for non-parametric procedures (31,32). In order to verify the differences between the groups, an analysis of variance (ANOVA) was carried out. GraphPad software (GraphPad Software, Incorporation, San Diego, CA, USA) was used and a value of p<0.05 was considered statistically significant. In these cases, the correlations among the minimal differences of analyzed groups were obtained through the Tukey test.

Results

ED₅₀ determination. Daily administrations of 25 mg/kg naringin (TN25) in rats inoculated with tumor cells led to more than 60% tumor growth inhibition in comparison with rats of the T group (Figure 1). Moreover, from the 16th day after tumor inoculation, tumor growth stabilized, and two animals presented tumoral regression, indicating that 25 mg/kg was an effective dose. Although the treatments with 10 and 35 mg/kg showed slight inhibition of tumor growth, these results were not statistically significant. In relation to weight gain, the TN25 group presented similar values to those of rats in the C group, indicating that naringin was capable of preventing cachexia (Figure 2).

Survival. A significant survival increase was observed in rats bearing W256 tumor treated with daily administration of naringin at 10 and 25 mg/kg (TN10 and TN25, respectively), resulting in 35% and 50% (p<0.05) of survival, respectively, in contrast to 100% mortality observed in the T group (Figure 3).

Hepatic and tumoral levels of TNF-α and IL-6. This study showed that naringin was effective in maintaining the TNF-α and IL-6 levels similar to those found in C group. The T
group showed high levels of these cytokines in both liver and tumor samples (Figure 4). The 25 mg/kg naringin treatment reduced TNF-α and IL-6 levels by about 49% and 44%, respectively, in tumor samples (Figure 4).

Discussion

In this work, we describe an in vivo effect of naringin in the therapeutic treatment of rats bearing W256 tumor. Naringin was shown to have a high antitumoral potential, demonstrated by a decrease in the tumor weight (more than 60%) and a complete tumor regression in two animals, in the presence of this flavonoid. In a previous study (39), we observed that the flavonoid quercetin had also anticancer potential, by inhibiting the tumor growth by 50% and increasing the survival of rats with tumor by 25%. In addition, we observed here that daily treatment with naringin maintained weight gain similar to that in C group. TNF-α and IL-6 levels were also similar to those found in control rats. These results indicate that naringin might act in the signaling pathway by inhibiting the release of these cytokines. The loss of adipose tissue, typical of cachexia, associated with cancer is partially related to the suppression of lipoprotein lipase activity by TNF-α (33), leading to a reduced uptake of exogenous lipids by adipocytes and an increased circulating triglyceride levels. Thus, the inhibition of cachexia by treatment with naringin may promote the maintenance of lipoprotein lipase activity by reducing TNF-α levels. A recent study showed that TNF-α can induce uncoupling of oxidative phosphorylation (34).

TNF-α may also be involved in the cancer-related anorexia, possibly by increasing levels of corticotropin-releasing hormone, a neurotransmitter in the central nervous system that suppresses hunger. Inui reported that the increase of TNF-α and IL-6 serum levels was associated, in some patients, with some tumor progression, but chronic administration of these cytokines, alone or combined, could reduce food intake, causing cachexia (35). High circulating IL-6 levels are associated with weight loss in some patients with lymphoma, lung and colon cancer; IL-6 levels were found to be higher in animals with W256 tumor (36).
However, in our study, animals treated with naringin had TNF-α and IL-6 levels similar to those observed in control animals.

This work demonstrated the great effectiveness of naringin as an antitumoral agent, confirmed through the data obtained from the experimental model of W256 tumor. The inhibition of tumor growth prolonged survival rate and prevented cachexia in animals treated with naringin. While these results highlight naringin as a potent antitumoral agent, further clinical studies will be necessary to define the precise role of this compound in cancer cachexia and the possible contributions of other related factors.

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