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Biological activities of Ginkgo extracts

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Abstract

The biological activity of methanolic the extracts of leaves, roots, leaf-derived callus, root-derived callus, ginkolide A, ginkgolide B, bilobalide and a commercial Ginkgo product (Tanakan®) was assessed. Bioassays consisted of the *Agrobacterium tumefaciens*-induced potato tumor assay and a Kirby-Bauer microbial sensitivity assay with pure strains of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pyogenes.* Methanolic extracts of leaves, leaf-derived callus, root-derived callus, bilobalide and Tanakan inhibited tumor formation significantly, but more weakly than the positive control, camptothecin. No activity against *E. coli* was detected, but extracts from both callus types inhibited the growth of *K. pneumonia, P. aeruginosa*, *S. aureus*, *S. epidermidis* and *S. pyogenes*. All extracts and reference compounds inhibited the growth of *S. pyogenes*. Leaf and root tissues contained the highest levels of ginkgolide A, as compared to the callus tissues; leaf tissue contained more of all three marker compounds than the callus tissues.

Keywords: Ginkgo extracts; Ginkgolides; Bilobalide; Callus cultures; Antitumoral and antimicrobial activity

Introduction

Ginkgo biloba L. is considered a living fossil due to its survival over millions of years (Braquet, 1988). It survived without structural modifications for over 200 millions years, which may be due to the fact that Ginkgo contains a number of biologically active compounds for its defense and can thus resist insects, bacteria and fungi (Bombardelli et al., 2000). Ginkgo extracts are reported to have antiparasitic, antifungal, antibacerial and antiviral activities (Atzori et al., 1993). Phenolic compounds isolated from sarcotesta of *Ginkgo biloba* showed antitumor activity against Sarcomar 180 ascites in mice (Itokawa et al., 1987, 1989). One of the Ginkgo terpenes, bilobalide, showed strong antiparasitic activity against *Pneumocyctic carinii* and other pathological strains (Bombardelli and Ghione, 1993).

Bioassays are adaptable for the purpose of screening and testing plant extracts (McLaughlin et al., 1998). Extracts are screened initially for biological activity and may be fractionated with subsequent identification of active ingredients. The Potato Tumor Induction assay was developed by Anand and Heberlein (1997) and used in screening of plant extracts for antitumor activity (McLaughlin et al., 1993). This assay utilizes a system based on the Crown Gall disease of plants caused by *Agrobacterium tumefaciens*. The bacterium contains a large plasmid (tumor inducing plasmid, Ti) which transforms wounded cells into autonomous tumor cells (McLaughlin, 1991). Coker et al. (2003) showed that the

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Potato Tumor Induction assay would detect antineoplastic activity of standard chemotheropeutic drugs regardless of the mechanisms of drug action. In 1980, Galsky et al. first demonstrated that the inhibition of Crown Gall tumors on potato (*Solanum tuberosum* L.) discs showed an apparent correlation with compounds and plant extracts known to be active in the 3PS (*in vitro* murine leukemia) assay, the standard test for antitumor agents. The Kirby-Bauer Disk Diffusion Assay measures antimicrobial activity based on bacteriostatic/bacteriocidal properties (Prescott and Harley, 1990). Filter paper discs impregnated with antibiotics are placed onto a medium inoculated with the test bacterium and incubated for 18–24 h at 37 °C. At the end of the incubation period, clear zones around the disk indicate either microbial resistance or sensitivity to the test chemical or extract, and are compared to values obtained with standard antimicrobial drugs (McCutcheon et al., 1992).

The objective of this study was to investigate the antitumor and antibacterial activity of Ginkgo extracts from leaf, root and callus tissues and of a commercial Ginkgo product.

Materials and methods

Plant material

Seeds were collected from *Ginkgo biloba* trees growing on the Clemson University campus in November of 1997, 1998 and 1999 (Cultivated specimen, Clemson University campus, CLEMS # 58453). The outer fleshy layer was removed. Seeds were washed with tap water and then soaked in 20% Clorox (6.0% sodium hypochlorite) for 10 min. After air drying, seeds were stored at 4 °C. Seeds were surface disinfested in 20% Clorox containing 2–3 drops of Tween 20 solution (Fischer Scientific, NJ) for 20 min and then rinsed three times in sterile distilled water. Each embryo was removed, placed in a sterile 95 × 25-mm capped shell vial, containing 10 ml of medium and incubated at 25 °C in a culture room under a light intensity of 22–28 $\mu mol/m^{-2}s^{-1}$ and a 16-h-photoperiod.

Leaves and roots were obtained from 8-week-old plants grown under aseptic conditions. Leaf discs were excised using a cork borer (4-mm diameter). Roots were cut into 4-mm lengths. Explants were placed in sterilized Petri dishes (Polystyrene 100 × 15 mm, VWR Scientific, PA) containing 25 ml of medium supplemented with growth regulators (Boonkaew, 2001). Callus (3 g) from the fourth subculture (16 weeks on a 28-day subculture interval) provided inoculum. Callus was grown for 28 days and harvested for extract preparation.

Plant material was dried immediately after harvesting for 18 h at 60 °C and then ground in a Wiley Mill with a 2-mm diameter mesh. Ground material (5 g) was extracted in 20 ml of methanol for 24 h, repeated three times. Combined crude methanolic extracts were filtered, evaporated to dryness and then reconstituted in 2.5 ml of methanol and filtered through a 0.2-μm filter. Reference standards were purchased from Sigma Co., St. Louis, USA: ginkgolide A, 90% purity, ginkgolide B, 90% purity; bilobalide, 95% purity. A commercial standardized Ginkgo leaf extract (Tanakan, Ispen-Beaufour) was also included in the assays.

Antitumor assay

Red potatoes (*Solanum tuberosum* L.) were disinfested by scrubbing under running water with a brush, then immersed in 10% Clorox (6.0% sodium hypochlorite) for 20 min. Potatoes were removed from the Clorox, blotted on sterile paper towels, and two sides were excised to provide a flat surface, and then placed in 20% Clorox for 15 min. Cylinders were cut from the disinfested sections with a sterile cork borer (10-mm diameter). Each cylinder segment was placed in sterile distilled water. After rinsing, each end of the cylinder was removed and the remaining cylinder was rinsed again in sterile distilled water. Discs were cut aseptically from the cylinders (approximately 0.5 cm in thickness) and placed in a 24-well culture plate containing 15% water agar. *A. tumefaciens* was grown on yeast extract media (YEM) for 48 h at 28 °C. Suspensions of *A. tumefaciens* in phosphate buffered saline (PBS) were standardized to 1×10^9 Colony forming units (CFU) as determined by an absorbance value of 0.96 ± 0.2 at 600 nm. A known tumor suppressant, Camptothecin (Sigma Co., St. Louis, USA), served as a positive control. Discs were also treated with methanol and an *A. tumefaciens* suspension as controls. Extracts and controls were prepared as followed; samples: 400 μl methanolic extract plus 100 μl water plus 500 μl *A. tumefaciens*; inhibitory control: 400 μl camptothecin plus 100 μl water plus 500 μl *A. tumefaciens*; *A. tumefaciens* control: 400 μl methanol plus 100 μl water plus 500 μl *A. tumefaciens*. Solvent control: 400 μl methanol plus 100 μl water plus 500 μl PBS. PBS replaced *A. tumefaciens* in solvent control to ascertain if the buffer would produce tumors on potato discs spontaneously without transformation with *A. tumefaciens*. The final concentrations of plant extracts and camptothecin were 50 mg and 20 μg, respectively.

Each disc was overlaid with 50 μl of extract/water/bacteria mix and incubated at room temperature for 12 days. On day 12 the discs were stained with Lugol's reagent (I_2KI; 5% I_2 plus 10% KI in distilled water). The starch in the potato tissue turned a dark-blue to