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Isolation and characterization of gallic acid and methyl gallate from the seed coats of *Givotia rottleriformis* Griff. and their anti-proliferative effect on human epidermoid carcinoma A431 cells

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ABSTRACT

Gallic acid (GA) and its derivative methyl gallate (MG) are well studied plant phenolics. They have exhibited anticancer effects in several cancer cell lines. However, the presence of GA/MG in the seed coats of *Givotia rottleriformis* and their inhibitory effect on human epidermoid carcinoma (A431) skin cancer cells were not reported. In this study we have isolated and chemically characterized the bioactive compounds GA and MG from the bioassay guided methanolic (MeOH) seed coat extracts of *G. rottleriformis*. The fractions obtained from open silica column chromatography were subjected to in vitro enzymatic assays. Among seven fractions we found that only fractions 5 and 6 showed significant inhibition activity toward COX-1 with an IC_{50} value of 28 μg/mL and 9.3 μg/mL and COX-2 with an IC_{50} value of 35 μg/mL and 7.0 μg/mL respectively. However, we could not find 5-LOX enzyme inhibition activity. MG (10 mg/g DW) and GA (6 mg/g DW) were the major compounds of seed coats. Cell viability was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, which showed that GA/MG significantly reduced the growth of A431 cells with an IC_{50} value of 25 μg/mL and 53 μg/mL and 11 μg/mL and 43 μg/mL at 24 h and 48 h, respectively. However the cytotoxic effect of GA/MG on HaCaT normal skin keratinocyte cell line was found to be less. Western blot analysis has shown that GA/MG treatment down regulated Bcl-2 and up regulated cleaved caspase-3 with respect to increasing doses. Our results conclude that GA and MG have potential anticancer effects and can be used as therapeutic agents for skin cancers.

1. Introduction

Skin diseases are becoming more common worldwide, because of increasing environmental pollutants, such as chemical hazards and radiations. Diverse factors influence skin cancers, e.g. solar radiation, UV irradiation. More than one million new skin cancer cases were reported annually in USA [1]. Increasing incidence of skin cancers was reported in AIDS (Acquired immune deficiency syndrome)

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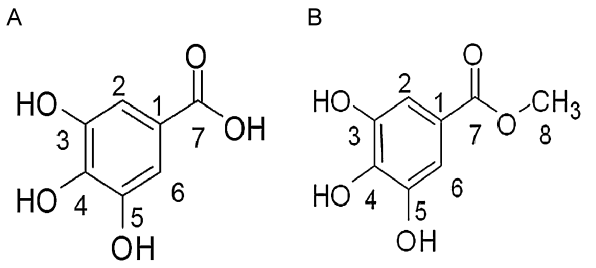

Fig. 1. Chemical structure of (A) gallic acid and (B) methyl gallate.

patients [2]. The cancer chemoprevention by natural agents such as phytochemicals, minerals and vitamins were shown to have effective results on various malignancies [3,4]. The curcumin isolated from plants inhibits UV-irradiation-induced oxidative stress and enhanced apoptosis in A431 cells [1,5]. The isolated resveratrol from plants was reported to have potential anti-cancer effect that enhanced apoptosis in A431 cells [6].

In this study we used the seed coats of *Givotia rottleriformis*, a tree species that belongs to Euphorbiaceae family. This plant grows in limited areas and particularly in the forests of Andhra Pradesh, Karnataka, Tamil Nadu and West Bengal in India. In our previous study we have observed that the seeds of this plant were rich in phenolics [7] and were reported to have anti-rheumatic, anti-psoriatic and anti-dandruff medicinal properties [8].

The gallic acid is isolated from plants [9,10] and the antioxidant and anticancer effect of GA were well reported in most of the cancer cells [9,11–17]. Many of GA derived chemical derivatives were also shown to have good anticancer properties [18]. Methyl gallate, a methyl ester of GA, was also isolated from several plants [19,20]. The biosynthesis of GA and MG takes place via dihydroshikimate, a derivative of phenylpropanoid biosynthesis [21]. The anticancer and anti oxidant effects of MG was reported in different cancer cells [22–27]. In addition MG was also shown to have anti-bacterial and anti-viral properties [28–30].

Cyclooxygenases (COX-1 and COX-2) and lipoxygenases (5-LOX, 12-LOX, 15-LOXa and 15-LOXb) are the key enzymes of arachidonic acid (AA) metabolism [31]. Previous reports suggested that inducible form of COX-1 and COX-2 leads to the biosynthesis of prostaglandins and thus causes inflammation and cancer [32–34]. Inhibition of 5-LOX blocks production of 5-LOX metabolites and triggers apoptosis in prostate cancer cells [35]. Therefore, identification of natural dual COX-2/5-LOX inhibitors is an interesting area of research to control cancer progression [36].

The over expression of Bcl-2 protein causes reduction of apoptosis in cancer cells [37–40]. In contrast, the up-regulation of cleaved caspase-3 activated by other caspases [41,42], enhances apoptosis and thus reduces cancer cell survival [43–45].

In the present study, we have isolated GA and MG from the seed coats of *G. rottleriformis* using chromatographic techniques and chemically characterized them using IR, NMR and LC–MS analysis (Fig. 1). The inhibitory effect of GA or MG on COX-1, COX-2 and 5-LOX was determined. We further studied the cytotoxic effect of purified GA and MG on human epidermoid carcinoma (A431) skin cancer cell line and normal human skin keratinocyte cell line HaCaT using MTT assay. The apoptotic effect of GA/MG was studied by western blotting analysis of Bcl-2 and cleaved caspase-3 in a dose dependent manner.

2. Materials and methods

2.1. General procedure

IR spectra was determined in the KBr pellet using a JASCO FT-IR model 5300 spectrophotometer with polystyrene as reference. NMR spectra were recorded at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR on Bruker-Avance-400 spectrometer with chloroform-d as solvent and tetramethylsilane (TMS) as reference ($\delta = 0$ ppm) in DMSO-d6 at 25 °C. The chemical shift was expressed in δ, downfield from the signal of internal TMS. Mass spectra were recorded using LC–MS-2010 (Shimadzu).

2.2. Plant materials

Mature and dry seeds of *G. rottleriformis* were collected during summer from Regional Forest Research Centre (RFRC), Rajahmundry, Andhra Pradesh, India.

2.3. Cell lines and reagents

A431 skin cancer cell line and normal HaCaT skin cell line were obtained from National Centre for Cell Science (NCCS), Pune, India. Dulbeco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), penicillin and streptomycin were purchased from GIBCO, Ltd. (BRL Life Technologies, Inc., Grand Island, NY). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] and dimethyl sulfoxide (DMSO) were obtained from Sigma; poly-L-lysine, glutaraldehyde, proteinase inhibitor K, propidium iodide (PI), phenyl methyl sulfonyl fluoride (PMSF), luepeptin, aprotinin, pepstatin A, trypsin, Tween 20, Triton X-100, TMPD (*N,N,N,N′*-tetramethyl-*p*-phenylenediamine) were purchased from Sigma Chemical Company (St. Louis, USA). The primary antibodies for Bcl-2 and cleaved caspase-3 were obtained from Upstate Biotechnology (Charlottesville, VA, USA). The GA and MG were purified from the seed coats of *G. rottleriformis*. All other chemicals and reagents used in the study were obtained either from Merck or Sigma.

2.4. Extraction and reverse phase HPLC analysis of crude extract

The seed coats were macerated into fine powder and were extracted in methanol (MeOH). The crude extract was filtered using Whatman No.1 filter paper and was concentrated using Rotavapor (R3, Buchi). The extract was further analyzed with RP-HPLC (Shimadzu) using C18 reverse phase column (Shim-pack column with dimensions 250 mm × 4.6 mm, particle size 5 μM), with flow rate of