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C-Glycosylated flavonoids from black gram husk: Protection against DNA and erythrocytes from oxidative damage and their cytotoxic effect on HeLa cells

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ABSTRACT

C-Glycosyl flavones are present in different plant tissues and they exhibit health benefits. In the present study, it was found that C-glycosyl flavones are distributed in different milled fractions of black gram and among these fractions, husk had the highest content of C-glycosyl flavones. Two C-glycosyl flavones from black gram husk were extracted and purified by preparative high-performance liquid chromatography (HPLC) column. The purity of each compound was assessed by analytical C18 column. The structure of each compound was confirmed by LC–MS/MS, NMR. The molecular mass of these compounds were found to be $[M-H]^-$, m/z 431.36 and $[M-H]^-$, m/z 431.35 and were identified as vitexin and isovitexin, respectively. Content of vitexin and isovitexin in aqueous ethanol extract was found to be 76 and 65 mg/g of extract, respectively. These C-glycosyl flavones protected DNA and erythrocytes from oxidative damage. The IC_{50} values for vitexin, isovitexin and quercetin for hemolysis were 6, 5.7 and 2.37 μg, respectively. These compounds also triggered the process of apoptosis in HeLa cells by downregulating Bcl-2 level with the simultaneous upregulation of Bax and caspase-3 protein expression. Thus, C-glycosyl flavones from black gram husk protected DNA and erythrocytes from oxidative damage and exhibited anticancer activity.

1. Introduction

Excessive formation of reactive oxygen species (ROS) induces oxidative stress leading to cell damage that can culminate in cell death. It has been reported that free radicals and radical mediated oxidation play a role in many pathological processes. ROS are capable of oxidizing cellular proteins, nucleic acids and lipids. Lipid peroxidation is a free-radical mediated propagation of oxidative insult to polyunsaturated fatty acids, and its termination occurs through enzymatic means or by free radical scavenging by antioxidants [28,29]. Severe oxidative stress is caused due to imbalance between the antioxidative defence systems and the formation of ROS that may alter intracellular signalling processes.

Polyphenols are potent antioxidants found ubiquitously in plants and consumed in relatively high quantities in the human diet. Extensive work has been carried out to understand the health benefits of several classes of polyphenolic compounds, in particular flavonoids [26]. Epidemiological studies have suggested the associations between the consumption of flavonoids and flavonoid-rich foods and the incidence of coronary heart disease, various cancers, stroke and osteoporosis [12]. Rather than exerting direct antioxidant effects, these polyphenols shows beneficial properties by various mechanisms and it involves their cellular interaction and signalling pathways related machinery that mediate cell function under both normal and pathological conditions [26].

Polyphenols are the class of secondary metabolites which are apparently absorbed from the upper gastrointestinal tract by a number of mechanisms [25]. Flavonoid glucosides interact with the sodium dependent glucose transporters (SGLT1) and transported across the apical membrane of enterocytes [9,27]. Beneficial effects of flavonoids have been strongly demonstrated in *in vitro* as well as *in vivo* systems including human studies. Flavonoids are a broad class of plant secondary metabolites with low molecular weight, characterized by the flavan nucleus. Naturally occurring flavonoids usually exist as *O*- or *C*-glycosides of flavonoid moiety. The *O*-glycosides possess sugar substituents bound to a hydroxyl of aglycone, usually at 3 or 7 positions, whereas *C*-glycosides possess sugar groups linked to the carbon of aglycone usually at C-6 or C-8 by forming a C—C bond, giving them more resistance to acid hydrolysis [4]. On the other hand, flavone *C*-glycosides such as vitexin and isovitexin frequently occur in many edible or medicinal plants

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including mung beans, pigeon pea leaves, bamboo leaves and ficus deltoidea leaves as main constituents [5,8,15,30].

Black gram is widely used pulse crop in India and also in different parts of the world. Dehusked black gram (dhal) is used for various food preparations. During milling of black gram into dhal, husk is one of the major fractions in the by-product. Earlier report from our laboratory indicated that aqueous extract of black gram husk exhibited antioxidant properties [11]. In the present study, we have extracted the husk bioactive components using 50% aqueous ethanol and fractionated in to two peaks by preparative RP-HPLC. Compounds present in these peaks were identified as vitexin and isovitexin using NMR and LC–MS/MS, and determined their antioxidant activity, protection against oxidative damage of DNA and erythrocyte and cytotoxic efficacy in cervical cancer cells.

2. Materials and methods

2.1. Chemicals

λ-DNA was purchased from Bangalore Genei, India. Agarose, H_2O_2, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), dimethyl sulfoxide (DMSO) were purchased from Sisco Research Laboratories. $FeSO_4 \cdot 7H_2O$, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, ascorbic acid, Tris base, ethidium bromide (EtBr), ethylenediaminetetraacetic acid (EDTA), Enhanced Chemiluminescence solution (ECL), vitexin, isovitexin and quercetin were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA). Polyvinylidene fluoride membrane (PVDF) was purchased from pall corporation, (New York, USA). BCA protein assay kit was purchased from Thermo Fisher Scientific (Massachusetts, USA). All other chemicals used were of analytical grade.

2.2. Milling of black gram and separation of milled products

Black gram (10 kg) was pitted in Versatile Dhal Mill (designed and developed by CSIR-CFTRI, Mysore, India) mixed with 30 ml of oil, kept overnight for tempering and dried at 60 °C for 8 h. The black gram thus obtained after treatment was milled using Versatile Dhal Mill. Black gram was milled into cotyledon, seed coat, and mixture of germ, aleurone, seed coat powder, and plumule. The husk was further fractionated by air classification as described earlier [10].

2.3. Extraction and isolation of bioactive components from black gram husk using different solvents

Powdered black gram husk was packed in a glass column, retained for 2 h in hexane and later gradually eluted with hexane till the eluent became colourless. The material remained after complete removal of hexane was further soaked and eluted with different solvents sequentially with increase in polarity such as chloroform, acetone, ethyl acetate, ethanol, aqueous ethanol (50%) and finally with water. The each fraction thus obtained was concentrated under reduced pressure.

2.4. Detection and quantification of c-glycosyl flavones in BGBP fractions

C-Glycosyl flavones like vitexin and isovitexin in black gram milled fractions were separated and quantified by analytical reversed-phase C18 column (4.6 × 250 mm) using HPLC system (Agilent- Model 1200 series) according to the method described by Kim et al. [16] using a diode array detector. A gradient solvent system consisting of solvent A water: acetic acid (99:1) and solvent B methanol: acetic acid (99:1) was used as mobile phase at a flow rate of 1 ml/min for a total run time of 50 min. The gradient elution used was as follows: 90–65% A in 10 min, 65–58% A in 25 min, 58–25% A in 35 min, 25% A in 40 min, 25–90% A in 45 min and 90% A in 50 min. Quantitative determination of the eluted flavones was detected at 337 nm and known quantities of vitexin and isovitexin standards were used for identification and quantification.

2.5. Purification of vitexin and isovitexin of aqueous ethanol fraction by preparative HPLC

The chromatographic separation was performed according to the method described in Section 2.4, on a Shimadzu Prep LC8A Preparative Chromatography system equipped with SCL-10AVP system controller (Shimadzu). The preparative HPLC was performed on a Varian, Pursuit XRs 10 C18 preparative column (250 mm × 21.2 mm). The flow rate was 8 ml/min and the wavelength used for detection was 337 nm. The sample volume injected was 2 ml. Two flavone *C*-glycoside peaks were collected manually, concentrated and evaporated to dryness by rotary evaporator.

2.6. Identification of vitexin and isovitexin of aqueous ethanol fraction

2.6.1. Evaluation of purity of purified flavone C-glycosides by HPLC and their identification by LC–MS/MS

Manually collected *C*-glycosyl flavones in aqueous ethanol fraction were tested for their purity according to the method described in Section 2.4. HPLC–ESI–MS analysis was done on a Waters platform ZMD 4000 system composed of a micro ZMD mass spectrophotometer, a Waters 2690 HPLC and a Waters 996 photo diode array detector (Waters corporation, MA, USA). Data were collected and processed via a personal computer running Mass Lynx software version 3.1 (Micromass, a diversion of Waters corporation, MA, USA). The samples in 10 μl diluted aliquot were separated on a reversed phase C18 column (4.6 × 250 mm), using a diode array detector (operating at 337 nm). Solvent A water: formic acid (99:1) and solvent B methanol: formic acid (99:1) was used as mobile phase at a flow rate of 1 ml/min for a total run time of 40 min. UV–vis absorption spectra were recorded on-line during HPLC analysis. The following ion optics was used- capillary voltage 3.5 kV, cone voltage 100 V and collision voltage 10 V. The source block temperature was 120 °C and the desolvation temperature was 350 °C. ESI–MS was performed using argon as cone gas (50 l/h) and nitrogen as desolvation gas (500 l/h). The electron spray probe flow was adjusted to 70 ml/min. Continuous mass spectra were recorded over the range of *m*/*z* 100–1000 with scan time 1 s and inter scan delay 0.1 s.

2.6.2. NMR analysis of flavone C-glycosides

^1H &^{13}C NMR spectra for the compounds were recorded on a Bruker Avance 500 MHz spectrometer (Bruker biospin, Reinstetten, Germany) using CD_3OD solvent.

2.7. Determination of free radical-scavenging activity

The effect of two isolated *C*-glycosyl flavones on DPPH radical scavenging activity was determined using earlier described method [1]. A 100 μM solution of DPPH in methanol was prepared and vitexin and isovitexin (200 μl) containing 5–30 μg were mixed with 1 ml of DPPH solution. The mixture was shaken vigorously and left in the dark at room temperature for 20 min. The absorbance of the resulting solution was measured at 517 nm. The control contained all the reagents except compounds/ascorbic acid. The capacity to scavenge DPPH radical was calculated by following equation:

$$\text{Scavengingactivity}(\%) = 1 - (A_s/A_0) \times 100$$

where A_0 is the absorbance at 517 nm of the control and A_s is the absorbance in the presence of compounds/ascorbic acid. The results