SUPPLEMENTARY MATERIAL

Natural Products from *Cuscuta reflexa Roxb.* with anti-proliferation activities in HCT116 Colorectal Cell Lines

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ABSTRACT: Parasitic *Cuscuta reflexa* Roxb. possesses many medicinal properties and is a rich source of a variety of biologically relevant natural products. Natural products are the prime source of leads, drugs, and drug templates and many of the anticancer and antiviral drugs are either based on natural product or derived from them. Cancer is a devastating disease and one of the leading causes of death worldwide despite improvements in patient survival during the past 50 years; new and improved treatments for cancer are therefore actively sought. Colorectal cancer is the fourth most prevalent cancer worldwide and is responsible for nearly 9% of all cancer deaths. Our search for anticancer natural products from *Cuscuta reflexa* has yielded four natural products: Scoparone (**1**), *p*-coumaric acid (**2**), stigmasta-3,5-diene (**3**) and 1-*O*-p-hydroxycinnamoylglucose (**4**) and among them 1-*O*-p-hydroxycinnamoyldlucose (**4**) showed promising anti-proliferative activities in HCT116 colorectal cell lines whereas compound **1**, **2** and **3** showed moderate activities.

Keywords: *Cuscuta reflexa, natural products, colorectal cancer cell line, HCT116*

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2. Experimental

2.1. Collection and Identification

The plant material (5 Kg) of *Cuscuta reflexa (*hosted by *Ficus corica)* was collected from Bagh district of Azad Jammu and Kashmir and identification was confirmed by Professor Dr. Muhammad Qayyum Khan, Department of Botany at the University of AJK where a voucher specimen was kept in the herbarium. HCT116 cells were maintained in DMED with 10% FBS at 37^0C in 5% CO_2.

2.2. Isolation and Characterization

Five kilograms of plant material was dried under shade and ground into powder which weighed 1.3 Kg. The dried powder was extracted thrice with methanol (7 liters each time) by mechanically stirring for 24 hours. The filtrates were concentrated under reduced pressure which gave 80 grams crude extract. The crude extract was fractioned into hexane, ethyl acetate, and butanol soluble parts. The ethyl acetate fraction (16.5 gm) was subjected to column chromatography using silica gel as the stationary phase. The column was eluted with a gradient system of hexane and ethyl acetate which lead to the purification of four natural products.

2.3. Isolation and Characterization of Natural Products

2.3.1. Scoparone (1), 6,7-Dimethoxy-2H-chromen-2-one (experimental name: MR-CR-AA-2)
The ethyl acetate fraction was chromatographed over silica gel with a gradual increase of polarity using hexane and ethyl acetate and fraction # 45 which eluted with hexane:ethyl acetate (85:15) gave pure compound **1** (11 mg) as amorphous powder. The purity of this compound was confirmed by TLC in hexane:ethyl acetate (75:25) (R_f ~ 0.6). It was UV active at 254 nm and was soluble in CH_2Cl_2. The molecular formula $C_{11}H_{10}O_4$ was deduced by molecular ion peak at m/z = 206.0 (100%) in EIMS and comparison of its ^{1}H-NMR spectrum with literature reference enabled to deduce the structure of this compound as 6,7-dimethoxy-2H-chromen-2-one (scoparone) (Yang, Lee et al. 2010).

2.3.2. p-Coumaric acid (2), (E)-3-(4-hydroxyphenyl)-2-propenoic acid (experimental name: MR-CR-AA-4)
The polarity of above column was further increased and sub-fractions eluted with hexane: ethyl acetate (70:25) were found to contain pure compound **2** (6 mg). It was active under UV light 254 nm and was soluble in acetone. Its purity was confirmed on TLC using CH_2Cl_2:MeOH (9:1) (R_f ~ 0.5). The molecular formula $C_9H_8O_3$ was deduced by molecular ion peak at *m/z* = 164.1 (100%) in EIMS and comparison of its ^{1}H-NMR spectrum with literature reference enabled to deduce the structure of this compound as (*E*)-3-(4-hydroxyphenyl)-2-propanoic acid (*p*-coumaric acid) (Yao, Lin et al. 2005).

2.3.3. Stigmasta-3,5-diene (3) (experimental name: MR-CR-AA-6)
The polarity of above column was further increased and sub-fractions eluted with hexane: ethyl acetate (50:50) were found to contain pure compound **3** (7 mg). It was active under UV light 254 nm and was soluble in pyridine and purity was confirmed on TLC using CH_2Cl_2:MeOH (8.5:1.5) (R_f ~ 0.5). Its molecular formula was deduced as to be $C_{29}H_{48}$ by EIMS 396.4 and the comparison of its ^{1}H-NMR spectrum with literature reference enabled us to deduce its structure as stigmasta-3,5-diene) (Cert, Lanzón et al. 1994).

2.3.4. 1-O-p-Hydroxycinnamoylglucose (4) (experimental name: MR-CR-AA-7)
The polarity of above column was further increased and sub-fractions eluted with hexane: ethyl acetate (5:95) were found to contain pure compound **4** (15 mg). It was visible in UV light 254 nm and was soluble in methanol. Its purity was confirmed on TLC using ethyl acetate:MeOH (9.5:0.5) (R_f ~ 0.4). Its molecular formula was deduced as $C_{15}H_{18}O_8$ by EIMS 326.1 and comparison of ^{1}H-NMR spectrum with literature reference enabled us to deduce the structure of this compound as 1-*O*-p-Hydroxycinnamoylglucose (Jiang, Hirose et al. 2001).

2.4. Cell Proliferation Assay
Compounds were assessed for their anti-proliferative activities in HCT116 colorectal cell lines using sulforhodamine B (SRB) proliferation assay as described elsewhere.(Vichai and Kirtikara 2006) Briefly, 2000 cells were seeded in 96 well-plates on day one. Next day, compounds diluted in the culture medium were added to the cells and incubated at 37°C and 5% CO_2 for 3 days. At the end of incubation, cells were fixed for a minimum of 2 hours at 4^{o}C with TCA (Trichloroacetic acid; 3% final concentration). Plates were washed with water, dried and stained with 0.06% SRB-solution for at least 30 minutes. Stained plates were washed with 1% acetic acid, air-dried overnight and incubated with 100 μL of 10 mM Tris pH 10.5 on a shaker for 5 minutes. Optical density was measured at a wavelength of 490 nm using a plate-reader and analysis performed to calculate the percentage growth inhibition or GI_{50} using GraphPad PRISM.

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