SUPPLEMENTARY MATERIALS

Antiproliferative and antiviral activity of methanolic extracts from Sardinian Maltese Mushroom (*Cynomorium coccineum* L.)

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Abstract

Cynomorium coccineum is a non-photosynthetic plant that grows in Mediterranean countries and that is amply used in the traditional medicine. The aim of this study was to extend previous studies on the chemical and biological properties of *C. coccineum,* evaluating the potential antiviral and antiproliferative activity of the methanolic extract. The MTT assay was used for the *in vitro* cytotoxic studies against human cancer-derived cell lines, while both MTT and plaque reduction (PRT) methods were used to evaluate the potential inhibitory effect of the extract against a panel of mammal viruses. The results obtained showed no selective activity against any DNA and RNA virus but revealed an interesting antiproliferative activity against human leukaemia-derived cell lines.

Experimental

Plant material

Fresh stems of *C. coccineum* were collected in south western Sardinia (Italy), in April 2017. The specimens were identified by Dr. Andrea C. Rinaldi, naturalist and mycologist, full professor at the Faculty of Biology and Pharmacy of the University of Cagliari (Rinaldi & Rescigno 2015). Reference material for stems (AR-CC-2017/4/1) was deposited in the collection of the Department of Biomedical Sciences, University of Cagliari. The specimens were kept cool and transferred to the laboratory within one hour from harvest. In a typical collection round, the stems (500 g) were gently cleaned to remove earthy residue and then dried with a blotter. The specimens were cut into slices having a thickness of about 0.5 cm and hung up to dry in air flow at 45 °C for 12 h in a vegetable dehydration machine (Rommelsbacher ElektroHausgeräte GmbH, Dinkelsbühl, Germany). After drying, the weight of the dried material was about 185 g.

Extraction of plant material

Two steps of extraction were performed as previously described (Rosa et al. 2012). Briefly, in the first one, dried and ground *C. coccineum* material was extracted with supercritical CO_2 (SFE) at 40 °C and 250 bars for 4 h. The second extraction step involved the use of a highly polar solvent on the same matrix in order to obtain a polar fraction. To this end, the exhausted *C. coccineum* powder was subjected to maceration by using methanol at ambient temperature in a stoppered container with frequent agitation for a period of 72 h at 4 °C. The extracts filtered and the supernatants obtained were dried in a rotary vacuum evaporator. The methanol extract of *C. coccineum* was dissolved in DMSO at 79 mg mL^{-1}, and then diluted in culture medium.

Cells

Cell lines were purchased from American Type Culture Collection (ATCC). The human cell lines were the following: normal tissue cells (skin fibroblasts, CRL-7065), lung squamous carcinoma (SK-MES-1), prostate carcinoma (DU-145), hepatocellular carcinoma (Hep-G2), skin malignant melanoma (SK-MEL-28), nasopharyngeal carcinoma, wild type (KBWT), nasopharyngeal carcinoma, multidrug resistant infected

with a retroviral vector containing a full-length cDNA for the human MDR1 gene that conferred the full MDR phenotype (KBMDR), nasopharyngeal carcinoma, stepwise selection for resistance with increasing concentration of vincristine (KBV20C), acute T-lymphoblastic leukaemia (CCRF-CEM), and acute B-lymphoblastic leukaemia (CCRF-SB). Cell lines supporting the multiplication of RNA and DNA viruses were the following: CD4+ human T-cells containing an integrated HTLV-1 genome (MT-4); Madin Darby Bovine Kidney (MDBK) [ATCC CCL22 (NBL-1) *Bos taurus*]; Baby Hamster Kidney (BHK-21) [ATCC CCL10 (C-13) *Mesocricetus auratus*] and Monkey kidney (Vero 76) [ATCC CRL 1587 *Cercopithecus aethiops*].

All cell lines were grown at 37 °C in a 5% CO_2 atmosphere in their specific media in according to ATCC instructions in the presence of 5-10% foetal bovine serum (FBS), antibiotic and, where suitable, sodium pyruvate supplement. All cell cultures were maintained in exponential growth by periodically splitting high density suspension cultures (i.e. 106 mL^{-1}) or when cell monolayers reached sub-confluence. The absence of mycoplasma contamination was checked periodically by the Hoechst staining method.

Viruses

Viruses were purchased from American Type Culture Collection (ATCC), except for Yellow Fever Virus (YFV), and Human Immunodeficiency Virus type-1 (HIV-1). Viruses representative of positive-sense, single-stranded RNAs (ssRNA+) were: i) Retroviridae: the IIIB laboratory strain of HIV-1, obtained from the supernatant of the persistently infected H9/IIIB cells (NIH 1983); (ii) Flaviviridae: yellow fever virus (YFV) [strain 17-D vaccine (Stamaril Pasteur J07B01)] and bovine viral diarrhoea virus (BVDV) [strain NADL (ATCC VR-534)]; (iii) Picornaviridae: human enterovirus B [coxsackie type B5 (CV-B5), strain Ohio-1 (ATCC VR-29)], and human enterovirus C [poliovirus type-1 (Polio-1), Sabin strain Chat (ATCC VR-1562)]. Viruses representative of negative-sense, single-stranded RNAs (ssRNA-) were: (iv) Paramyxoviridae: human respiratory syncytial virus (RSV) [strain A2 (ATCC VR-1540)]; (v) Rhabdoviridae: vesicular stomatitis virus (VSV) [lab strain Indiana (ATCC VR 1540)]. The virus representative of double-stranded RNAs (dsRNA) Reoviridae was (vi) reovirus type-1 (Reo-1) [simian virus 12, strain 3651 (ATCC VR-214)]. DNA virus representatives were: (vii) Poxviridae: vacciniavirus (VV) [vaccine strain ElstreeeLister (ATCC VR-1549)]; (vi) Herpesviridae: human herpes 1 (HSV-1) [strain KOS (ATCC

VR-1493)].

Cytotoxicity assay

The cytotoxic effect of test compounds was evaluated in exponentially growing cell cultures. Stock solution of methanol extract was made at 79 mg mL^{-1} in DMSO and stored at 4 °C in the dark; the cyanidin 3-*O*-glucoside stock was prepared at a concentration of 1 mg mL^{-1} in DMSO, stored at -80 °C in the dark. For the evaluation of cytotoxicity, compounds were serially diluted in growth medium so that the concentration of DMSO was never higher than 0.1%. Suspension cell lines were seeded in 96-well plates at $1x10^5$ cells mL^{-1} in specific growth medium as described above, with or without serial dilutions of test compounds; adherent cell lines were seeded at $1x10^4$ cells/well and incubated overnight before the treatment with serial dilutions of compounds.

Cell viability was determined after 96 h at 37 °C by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method (Pauwels et al. 1988) as previously described by (Vascellari et al. 2019). Vincristine and doxorubicine were used as reference anticancer compounds.

Cytotoxicity tests were also performed in parallel with antiviral assay in virus-infected cells as previously reported (Carta et al. 2018; Vascellari et al. 2019). In brief, MDBK, BHK-21 and Vero-76 cells were seeded at $3x10^4$ cells/well, $5x10^4$ cells/well, $2.5x10^4$ cells/well, respectively, and incubated overnight before the treatment with serial dilutions of *C. coccineum* extract. After 24 h, in each well were added 50 µL of maintenance medium (apposite medium supplemented with 0.5% inactivated FBS) to make the conditions identical with respect to the antiviral activity assay. After 1 h in the dark at room temperature, 50 µL of maintenance medium, with or without serial dilutions of test extract, were added. Unless stated, Vero-76 viability was determined by the crystal violet staining method (Carta et al. 2018).

Antiviral assays

Extract activity against HIV-1, YFV, Reo-1 and BVDV was based on inhibition of virus-induced cytopathogenicity in MT-4, BHK-21 and MDBK cells, acutely infected with a multiplicity of infection (m.o.i.) of 0.01 as previously described by (Carta et al. 2018). Antiviral activity against CV-B5, Sb-1, VV, HSV-1 and RSV was determined by plaque

reduction assays (PRT) in infected Vero-76 cell monolayers (Carta et al. 2018).

References

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