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Original article

Antiestrogenic and antigenotoxic activity of bee pollen from *Cystus incanus* and *Salix alba* as evaluated by the yeast estrogen screen and the micronucleus assay in human lymphocytes

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ABSTRACT

The estrogenic/antiestrogenic activity and the genotoxicity/antigenotoxicity of bee pollen from *Salix alba* L. and *Cystus incanus* L. and its derivative extracts in yeast and human cells was investigated. All samples showed a marked inhibitory effect on the activity of the natural estrogen 17 β-estradiol (higher than 90% for extracts **2**) and failed to cause estrogenic activity and chromosome damage. At least one preparation from each species showed a marked antigenotoxic effect against the action of the anticancer drugs mytomicin C, bleomycin, and vincristine. Bee pollens from *C. incanus* and *S. alba* were found to be neither genotoxic nor estrogenic as well as effective estrogen inhibitors, and able to reduce the chromosome damage induced by the three cancer drugs used, thus supporting their use as a safe food supplement and future chemoprotective/chemopreventive agents.

1. Introduction

The consumption of bee pollen or its derivative products as a dietary supplement by humans has been rapidly increasing over the last decade due to its high saccharide, lipid, protein and amino acid contents. Bee pollen is also a very important source of vitamins and polyphenolic compounds, particularly flavonoids [1,2]. Some classes of these substances, similarly to natural estrogens (phytoestrogens), are able to bind intracellular estrogen receptors (ERs) thus causing estrogenic/antiestrogenic effects [3,4]. In addition, they exhibit a broad spectrum of biological effects which can be beneficial or harmful for human health. Flavonoids, in fact, beside having anti-inflammatory, antioxidant and anticancer activity, were proven to be mutagenic, pro-oxidant and/or pro-apoptotic in different *in vitro* cell systems, even though the effects are observed, in general, at high concentrations [5–7]. Clinical and epidemiological studies indicate that these compounds can also interact with chemotherapeutic drugs during cancer treatment [8,9]. Thus, considering that pollen preparations are generally made away from medical examination regardless of any drug interactions (which are often harmful), assessment of the potential genotoxic or estrogenic/antiestrogenic activity can be of particular relevance to the safety of any product intended for alimentary or medical use. At present, no such data are available in literature, with the exception of two works in which honeybee pollen showed no estrogenic activity [10] or antiestrogenic and antioxidant properties [11].

With this background in mind, we aimed at investigating clear bee pollen powder (BPP) collected from two Mediterranean species (*S. alba* L. and *C. incanus* L.) and its derivative extracts for estrogenic and antiestrogenic activity in a yeast reporter assay or for genotoxicity in human cells. The analysis of micronucleus (MN) formation in phytohaemagglutinin-stimulated and cytochalasin B-blocked human peripheral lymphocytes which detects chromosome breakage and/or chromosome malsegregation, is commonly used as a marker of genome damage [12]. To study its biological profile in more depth, we further investigated the ability of pollen to attenuate, in an antigenotoxicity assay, the chromosome damage

Abbreviations: BPP, bee pollen powder; MMC, mytomicin C; BLM, bleomicin; VCR, vincristine; 4-OHT, 4-hydroxytamoxifen; DBS, double strand breaks; CPI, cell proliferation index; MN, micronucleus(i); E_2, 17 β-estradiol; β-gal activity, β-galactosidase activity; ER, estrogen receptor.

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induced by bleomycin (BLM), mitomycin C (MMC) and vincristine (VCR), three anticancer drugs acting via different molecular mechanisms. BLM is activated to form site specific radicals which are able to directly generate single or double strand breaks (DSB), thus resulting in a potent mutagenic and cytotoxic agent [13]. MMC produce prevalently mono- and bifunctional alkylation of guanine residues that irreversibly bind to DNA to form interstrand and intrastrand DNA cross-links via a reductive metabolism [14]. VCR binds to a site on the tubulin subunits, inhibiting their polymerisation to form the mitotic spindle microtubules and possibly also the centriole microtubules, thus causing chromosome malsegregation [15]. These three mutagenic drugs are regarded as suspected human carcinogens, and VCR has been already included into group 1 carcinogens (carcinogenic to humans) by IARC [16].

2. Results

2.1. Botanical identification and phenolic compound content

Melissopalynological analysis showed that the most abundant species in *C. incanus* bee pollen was *C. incanus* L. (70%). Pollen grains from *Quercus ilex*, *Quercus* spp., *Asphodelus* spp., and family Brassicaceae were also present at a lower extent. The most abundant species in *S. alba* bee pollen was: *S. alba* more than 80%, Brassica L. 10% and at a lower extent, pollen grains of *Salici populetum nigrae* and *Quercus robur*.

Table 1 shows phenolic compound composition of extract **1** and extract **2** from *C. incanus* and *S. alba* BPP. In extract **1** of *C. incanus*, only pinocembrin and chrysin reached at least 1 μmol/g (total phenolic compound content = 4.28 μmol/g), while *S. alba* concentration of pinocembrin, chrysin, galangin were 3.30, 3.90 and 2.05 μmol/g, respectively (total phenolic compound content, 12.30 μmol/g). The most represented compounds of extract **2** were: isorhamnetin (6.70 μmol/g), quercetin (3.25 μmol/g), kaempferol (1.56 μmol/g) for *C. incanus* (total phenolic compound content, 13.80 μmol/g); coumaric acid (7.40 μmol/g), kaempferol (4.80 μmol/g), taxifolin (2.85 μmol/g) and caffeic acid (2.05 μmol/g) for *S. alba* (total phenolic compound content, 23.65 μmol/g).

2.2. In vitro estrogenic/antiestrogenic activity

Both BPP and the two derivative extracts of *C. incanus* showed a weak dose-dependent increase in estrogenic activity, however less than 20% with a maximum β-gal activity of 13.2% of 17 β-estradiol (E_2). Significant differences ($p < 0.01$) were seen between extract **2** and BPP or extract **1**. At the highest concentration tested (660 μg/ml), the E_2 relative activity was higher for extract **2** (10.76 ± 1.50%) than for extract **1** (7.32 ± 0.82%) or BPP (7.13 ± 0.03%). The results of the *in vitro* antiestrogenic activity are reported in Fig. 1 for *C. incanus* (panel A) and *S. alba* (panel B), respectively. Both BPP and the two derivative extracts of *C. incanus* showed a dose-dependent inhibitory effect on β-gal expression induced by 1 nM E_2. 4-hydroxytamoxifen (4-OHT) exhibited strong inhibitory effect (65.13% inhibition) on β-gal activity induced by E_2. As shown in Fig. 1A, under the conditions when 4-OHT inhibited the E_2-mediated activity by 65.13%, bee pollen inhibited the activity by 62.6 ± 2.64% (BPP), 62.6 ± 4.27% (extract **1**), and by 92.8 ± 4.21% (extract **2**), at a concentration of 660 μg/ml (about 200 fold higher). Also in this case, the strong inhibitory effect of extract **2** was significantly different ($p < 0.001$) from the effects observed for the two other pollen preparations.

All preparations from *S. alba* exhibited a moderate increase in the E_2-like activity never exceeding 20%. Estrogenic activity of extract **2** detected at 660 μg/ml (13.20 ± 2.06%) was significantly more elevated ($p < 0.001$), as compared to that of BPP (6.59 ± 0.02%) or extract **1** (6.28 ± 0.53%). The antiestrogenic activity of *S. alba* bee pollen samples are showed in Fig. 1, panel B. Once again, at final concentration of 660 μg/ml, extract **2** showed the highest inhibition value (91.82 ± 7.46%, $p < 0.01$) with respect to BPP (70.35 ± 3.34%) or extract **1** (78.61 ± 0.67%). At the highest concentration tested both extracts **2** strongly inhibited the yeast

Table 1
Phenolic compound content of *C. incanus* L. and *S. alba* L. bee pollen samples

	C. incanus L.		*S. alba* L.	
	Extract **1** (μmol/g)	Extract **2** (μmol/g)	Extract **1** (μmol/g)	Extract **2** (μmol/g)
Caffeic acid	0.35	0.60	1.30	2.05
Coumaric acid	ND	ND	1.30	7.40
Galangin	0.86	0.40	2.05	0.70
Isorhamnetin	ND	6.70	0.20	1.20
Kaempferol	0.30	1.56	0.25	4.80
Myricetin	ND	ND	ND	ND
Quercetin	ND	3.25	ND	ND
Luteolin	ND	ND	ND	1.65
Chrysin	1.35	0.79	3.90	1.80
Daidzein	ND	ND	ND	ND
Genistein	ND	ND	ND	ND
Naringenin	ND	ND	ND	0.65
Pinocembrin	1.42	0.50	3.30	0.55
Taxifolin	ND	ND	ND	2.85

ND = non detectable.

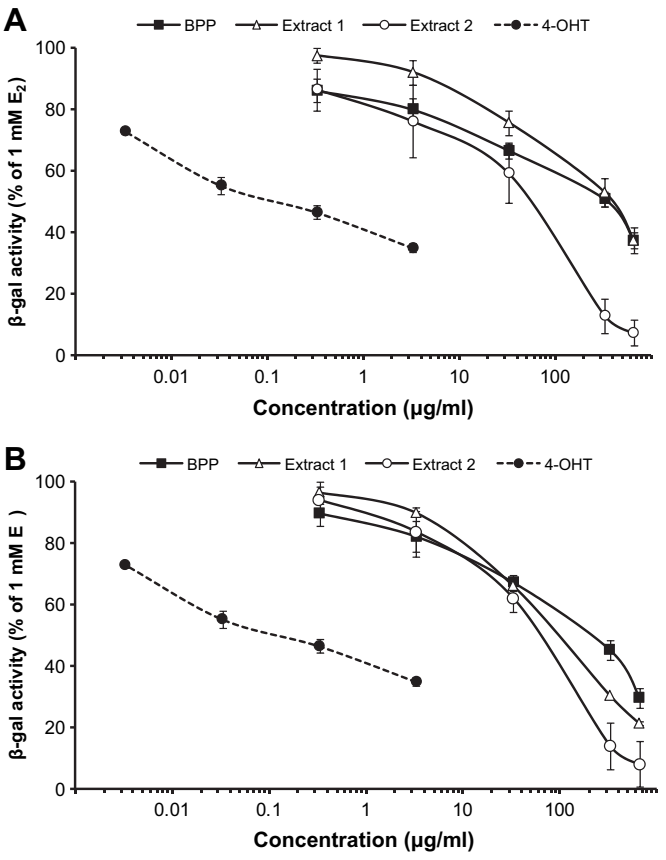

Fig. 1. Inhibitory activity of different concentrations of bee pollen samples from *C. incanus* L. (A) and *S. alba* L. (B), its derivative extracts and 4-hydroxytamoxifen (4-OHT) on β-gal activity induced by 1 nM E_2 in yeast. To test for antagonistic activity, the yeast strain was incubated with E_2 in the presence or absence of samples. The activity is expressed as a percentage of the β-gal activity induced by E_2 (100%). Values are the means ± SE.