Estrogenic activity of Red Cover (Trifolium Pratense L.) extracts and its relationship with isoflavone content: an *in vitro* study in MCF-7 cells

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Red clover (RCL) is a perennial plant rich in phytoestrogens (mainly biochanin A, daidzein, genistein and formononetin) which has been used to treat menopausal symptoms, as an alternative to hormone replacement therapy, and to prevent osteoporosis, as well as for prostatic hypertrophy (Ulbricht & Basch, 2005). A critical issue with botanical preparations is their chemical standardization and its relationship with the resulting biological activity (Ong, 2004). RCL extracts are standardized to their isoflavone content and, according to the United States Pharmacopeia (USP, Rockville, MD), the analytical reference for the preparation of standardized RCL extracts is the ratio 5,7-dihydroxyisoflavones (biochanin A + genistein) to 7-hydroxyisoflavones (daidzein + formononetin). Fluctuations in individual isoflavone content may indeed result from differences in extraction procedure as well as from seasonal variation (Booth et al., 2006). No information exists regarding the consequences of differences in the relative content of individual isoflavones on the biological activity of RCL extracts (Wang et al., 2008).

The aim of the present study was to assess the estrogenicity of different batches of RCL extracts and to assess their possible relationship with their content in isoflavones. To this end, we measured the *in vitro* effect of each extract on the proliferative response of the estrogen-dependent MCF-7 cells by means of flow cytometry after staining of DNA with propidium iodide taking the percentage variation of cells in the S phase of the cell cycle as an index of proliferation (Cosentino et al., 2007).

Estradiol (E2) and isoflavones contained in RCL concentration-dependently increased MCF-7 cell proliferation. E2 acted in the pM-nM concentration range, while isoflavones were active in the nM-μM range, with efficacies which were not significantly different from that of E2. The order of potency was: E2 >>> genistein > biochanin A = daidzein > formononetin. All the tested RCL extracts (indicated as A, B, C, D, E) increased the percentage of MCF-7 cells in the S phase of the cell cycle with similar efficacy and with significantly different potency. RCL extracts did not differ between each other according to their respective mean Emax, however, in comparison to E2, RCL extracts A and E had significantly higher Emax ($P<0.05$ in both cases). The order of potency of RCL extracts was: $A = B \geq C \geq D = E$. The potency of all but one RCL extracts was directly correlated with the respective ratios of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavones content, while the efficacy of all extracts showed a slightly significant inverse correlation with the content of genistein. The estrogen receptor antagonist 4-hydroxytamoxifen completely inhibited the response to E2 and to RCL extracts, suggesting that their effects on MCF-7 cell proliferation was attributable to estrogen receptor activation. The inhibitory potency of 4-hydroxytamoxifen towards the RCL extracts were not significantly different.

The present study showed that estrogenic activity of RCL extracts can be reproducibly assessed in cultured MCF-7 cells. RCL extracts can be differentiated according to their pharmacological potencies and - to a lesser extent - to their respective Emax. Moreover, the potencies of RCL extracts A, B, D and E (but not C) had a direct and highly significant correlation with the ratios of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavones. Additional observations are needed to confirm this finding as well as to establish the reason(s) why RCL extract C did not comply with such relationship. Finally, these results also support the use of MCF-7 cells as a suitable bioassay for estrogenicity testing of RCL extracts.

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