Phytomedicine 17 (2010) 895–901

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.de/phymed

Isoflavones isolated from red clover (*Trifolium pratense*) inhibit smooth muscle contraction of the isolated rat prostate gland

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ARTICLE INFO

Keywords:
Smooth muscle contractility
Genistein
Formononetin
Biochanin A
Chemical fractionation

ABSTRACT

This study investigated whether red clover contains any bioactive constituents which may affect contractility of rat prostatic smooth muscle in an attempt to determine whether its medicinal use in the treatment of benign prostatic hyperplasia is supported by pharmacological effects. A commercially available red clover extract was chemically fractionated and various isoflavones (genistein, formononetin and biochanin A) were isolated from these fractions and their effects on contractility were examined on preparations of the isolated rat prostate gland. Contractile effects of the isolated fractions were compared with commercially available isoflavones (genistein, formononetin and biochanin A). Pharmacological tools were used to investigate the mechanism of action modifying smooth muscle contraction. Crude red clover extract (Trinovin®) inhibited electrical field stimulation induced contractions of the rat prostate across a range of frequencies with an IC_{50} of approximately 68 μg/ml. Contractions of the rat prostate elicited by exogenous administration of acetylcholine, noradrenaline or adenosine 5′-triphosphate (ATP) were also inhibited. Chromatographic separation, and final purification by high performance liquid chromatography (HPLC) permitted the isolation of the isoflavones: daidzein, calycosin, formononetin, prunetin, pratensin, biochanin A and genistein. Genistein, formononetin and biochanin A (100 μM) from either commercial sources or isolated from red clover extract inhibited electrical field stimulation induced contractions of the isolated rat prostate. It is concluded that isoflavones contained in red clover are able to inhibit prostatic smooth muscle contractions in addition to their antiproliferative effects. However, the high concentrations required to observe these smooth muscle relaxant effects mean that a therapeutic benefit from this mechanism is unlikely at doses used clinically.

Introduction

The herbal preparation red clover (*Trifolium pratense*) is commonly used in the treatment of prostatic diseases such as benign prostatic hyperplasia (BPH) (Engelhardt and Riedl 2008). Red clover is a member of the leguminosae family which includes soy bean, pea and alfalfa. It is rich in isoflavonoids which chemically encompass a large group of structurally similar compounds based on a 3-phenylchromen-4-one bearing different substituents. There are thought to be as many as 1600 isoflavonoids, with the majority of these being isolated from the leguminosae family (Booth et al. 2006). Isoflavones are a subclass of isoflavonoids which accumulate in the vacuoles of legumes either as glycosides or in the 6′-O-malonated form (De Rijke et al. 2001). The glycoside and malonate forms are more soluble and more stable than the aglycones but generally lack biological activity (Piersen et al. 2004; Papadopoulos et al. 2006). The glycoside or 6′-O-malonated forms are labile during extraction processing and form aglycones that are relatively scarce in plant tissue (Coward et al. 1998). The major isoflavones existing as aglycones in red clover are formononetin and biochanin A, totalling 0.9% in dry forage (Wu et al. 2003). Liquid chromatography has been used as the primary method for separation of isoflavonoids from red clover with further identification and analysis of the various isoflavonoids carried out using reverse phase high pressure liquid chromatography (RP-HPLC) (Booth et al. 2006; Coward et al. 1998).

The physiological activity of red clover was discovered by Australian scientists in the 1940s, when it was found to have strong anti-estrogenic effects in sheep later attributed to high concentrations of isoflavones (Bennetts and Underwood 1951). In addition, Asian men typically enjoy good prostate health with lower cancer rates and less severe lower urinary tract symptoms (Breslow et al. 1977; Yu et al. 1991; Whittemore et al. 1995; Risbridger et al. 2001). This is thought to be a consequence of their diet which is typically high in phytoestrogens and isoflavones (Adlercreutz and Mazur 1997; Morton et al. 1997). Chronic *in vivo* studies in adult mice have shown that over a 28-day period, prostate size is reduced

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0944-7113/$ – see front matter.
doi:10.1016/j.phymed.2010.05.006

following oral administration of red clover. Furthermore, histological evaluation revealed an increase in apoptotic cells (Risbridger et al. 2001). In contrast, a recent human trial of 60 mg/day of red clover over a 1-year period in 20 men found very little difference in the overall international prostate symptom score (IPSS) versus placebo (Engelhardt and Riedl 2008). Nevertheless, red clover extracts are often marketed as having beneficial effects in the treatment of BPH which are attributed to its antiproliferative effects.

The most effective pharmacotherapies for BPH are based on α-adrenergic receptor blockade. This class of drugs act by relaxing prostatic smooth muscle to alleviate the symptoms produced by urethral obstruction. The α_1-adrenergic receptor antagonists like tamsulosin are the least costly, most effective and best tolerated pharmacological treatment for BPH (Hutchison et al. 2007). Furthermore, there is a poor correlation between lower urinary tract symptoms (LUTS) caused by BPH and prostate size (Eckhardt et al. 2001a,b,c). These two observations suggest that drugs which relax prostatic smooth muscle are more effective than drugs which act by shrinking the prostate. Recently, combination therapy using an α_1-adrenoceptor antagonist to relieve urinary symptoms and a 5α-reductase inhibitor to slow or halt disease progression has become more popular (Schulman 2003).

Isoflavones present in red clover have been shown to inhibit smooth muscle contractions in isolated guinea-pig ileum (Herrera et al. 1992), rat uterus (Revuelta et al. 1997) and guinea-pig gallbladder (Wang et al. 2008). Isoflavones present in red clover may therefore be able to produce prostatic smooth muscle relaxation which would have a beneficial effect in the treatment of BPH in addition to any antiproliferative effect that the herb may possess. The aim of this study was to investigate the effects of a commercially available red clover extract on the contractility of the rat isolated prostate gland. Functional pharmacological isolated organ bath experiments were used to investigate the contractile effects of red clover extract and any active constituents isolated through systematic fractionation by liquid chromatography.

Materials and methods

Red clover extract

The red clover preparation used in this study was Trinovin® (Novogen, Sydney, Australia) which is a commercially available standardised tablet containing 40 mg of isoflavones extracted from red clover. Trinovin® tablets were pulverised with a mortar and pestle, and the resulting powder (480 mg) was then suspended in absolute ethanol (5 ml) and filtered to give an ethanol solution suitable for use in isolated organ baths. Evaporation of this solution gave a green residue (230 mg). Commercially available formononetin, biochanin A and genistein were purchased from Sigma (Sydney, Australia). All isoflavones were dissolved in ethanol or dimethyl sulfoxide (DMSO) on the day of experimentation.

Isolation of isoflavones

Red clover extract from six tablets (ethanol extract as above, 3.0 g) was redissolved in ethanol (100 ml), and the solution added to silica gel 60 (25 g), and evaporated to dryness. The resulting powder was placed on top of a silica chromatography column, and eluted successively with hexane, chloroform, ethyl acetate and methanol (500 ml each), to give four fractions. The ethyl acetate fraction was further separated into individual compounds by preparative HPLC on a Waters Prep LC System or Waters 600 42 controller coupled to a Waters 486 Tunable Absorbance Detector using a Phenomenex Luna 5u C8 (2) 100 A (50 mm × 21.20 mm ID) column, eluting with gradients of solvent A (0.1% trifluoroacetic acid (TFA) in water) to solvent B (80% acetonitrile (AcCN) in water, 0.1% TFA). The biological activity of the individual isoflavone compounds obtained through this separation procedure was then compared to the activity of commercial isoflavone samples.

Spectroscopic analysis

All extract samples were subjected to analysis by ESI-MS on a Micro mass Platform II single quadruple mass spectrometer equipped with an atmospheric pressure (ESI/APCI) ion source. Sample management was facilitated by an Agilent 1100 series HPLC system and the instrument was controlled using Mass Lynx software version 3.5. ^{1}H NMR analysis was conducted on samples dissolved in *d* chloroform or d_6 acetone, as appropriate, on a Bruker Avance 300 MHz NMR spectrometer, referenced to residual proteo solvent, and spectra obtained for isolated isoflavones were compared with literature data or commercially available samples to confirm identity (Chang et al. 1995; Du et al. 2006; Hanawa et al. 2001; Tahara et al. 1994; Talukdar et al. 2000). Analytical HPLC was used to assess the purity of samples, and was conducted on a Waters 2690 Separation Module coupled with a Waters 996 Photodiode Array Detector with a Phenomenex Luna 5U C8 (2) 100 A (150 mm × 4.60 mm ID) column. Samples were run over a gradient of 0–100% Buffer B (Buffer A (H_2O, 0.1% TFA)), Buffer B (80% AcCN, 19.9% H_2O, 0.1% TFA). Empower Pro software managed the processing of the samples.

Animals and tissues

Male Sprague–Dawley rats were housed at 22 °C and exposed to a photoperiod of 12 h light/12 h dark. Rats were allowed access to food and water *ad libitum*. Rats were culled by cervical dislocation. An abdominal incision was made, exposing the male urogenital tract and the left and right lobes of the prostate were removed providing two prostate preparations from each rat. Ethical approval was obtained from the Monash University Standing Committee of Animal Ethics in Animal Experimentation (VCPA2006/9).

Isolated organ bath studies

The prostate lobes were placed in a Petri dish containing Krebs–Henseleit solution (NaCl 118.1 mM, KCl 4.7 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 25.0 mM, glucose 11.7 mM, $MgSO_4$ 0.5 mM and $CaCl_2$ 2.5 mM). The isolated tissues were mounted in 10 ml organ baths containing Krebs–Henseleit solution warmed to 37 °C and bubbled with 5% CO_2 in O_2. One end of the prostate was attached to an isometric Grass FT03 force–displacement transducer connected to a PowerLab data acquisition system run on a personal computer. The other end of the preparation was attached to a perspex tissue holder incorporating two parallel vertical platinum electrodes. Tissues were initially equilibrated for 60 min, under a resting force of approximately 0.7 g. During the 60 min equilibration period, nerve terminals within the tissues were field stimulated using the electrodes connected to a Grass S88 stimulator to deliver pulses of 0.5 ms, 60 V at 0.01 Hz. This was done to ensure tissue viability. The organ bath medium was replaced when frothing occurred, due to prostatic secretions.

Electrical field stimulation

Following the equilibration period, frequency–response curves were conducted to examine whether red clover extract affected nerve mediated contractile responses elicited by electrical field stimulation (0.5 ms, 60 V, 1–20 Hz). A frequency progression ratio of approximately one-third of a log unit was used. Pulses were delivered in 10 s trains at intervals of 10 min. At the completion of the frequency–response curve and after a further 15 min rest, tissues were washed and red clover extract or vehicle was added to the organ bath to incubate the tissue for 30 min prior to a sec-