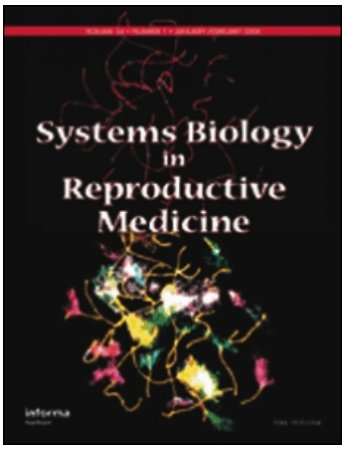

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Plants Used in Chinese Medicine for the Treatment of Male Infertility Possess Antioxidant and Anti-Oestrogenic Activity

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Research Article

Plants Used in Chinese Medicine for the Treatment of Male Infertility Possess Antioxidant and Anti-Oestrogenic Activity

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In this study Chinese herbs commonly used in the treatment of male infertility were investigated for relevant biochemical activity. Male factor infertility predominantly arises via barriers to, or defects in, spermatogenesis. The process of spermatogenesis is under strict endocrine control; in addition oxidative stress has been implicated in male infertility with significant levels of reactive oxygen species detected in 25% of infertile males. A total of 37 individual herbs and seven herb decoctions used in the treatment of male factor infertility were therefore tested for endocrine activity using a recombinant yeast based assay and antioxidant activity using the FRAP (ferric reducing antioxidant potential) assay. Individual herbs tested did not show androgenic properties, 20 showed strong and 10 weak anti-oestrogenic activity (per g of dried herb tamoxifen equivalents ranged from 1.18–1280.66 mg and 0.06–0.98 mg, respectively). Oestrogenic responses were elicited for two herbs (85.30–550 μg oestradiol equivalents/g dried herb), with seven and three herbs exhibiting a strong or weak anti-androgenic response (per g of dried herb DHT equivalents ranged from 1.54–66.78 mg and 0.17–0.32 mg), respectively. Of these 37 herbs, strong (15 herbs), intermediate (7 herbs) and weak/no (15 herbs) antioxidant activity was detected (ranging from 0.912–1.26; 0.6–0.88 and 0–0.468 μg ascorbate equivalent/mg dried herb, respectively). The seven decoctions (previously used to treat patients) tested elicited strong (5 herbs) and weak (2 herbs) anti-oestrogenic responses (per g of dried herb tamoxifen equivalents ranged from 1.14–13.23 mg and 0.22–0.26 mg, respectively), but not oestrogenic, androgenic nor anti-androgenic, consistent with their individual composition. With regard to antioxidant activity the following responses were recorded: three strong, three intermediate and one weak (ranging from 1.02–1.2; 0.72–0.76 and 0.44 μg ascorbate equivalent/mg dried herb, respectively). The prospects for introducing Chinese herbal treatments into the Western-based medicine are discussed.

KEYWORDS anti-oestrogen, antioxidant, FRAP, male infertility, traditional Chinese medicine

Abbreviations: CPRG: chlorophenol red-B-D-galactopyranoside; DHT: dihydrotestosterone; FRAP: ferric reducing antioxidant potential; FSH: follicle stimulating hormone; GnRH: gonadotrophin-releasing hormone; hAR: human androgen receptor gene; hER: human oestrogen receptor gene; HPG: hypothalamic-pituitary-gonadal axis; LH: luteinizing hormone; ROS: reactive oxygen species; TCM: traditional Chinese herbal medicine; TDS: testicular dysgenesis syndrome.

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INTRODUCTION

Alternative medicine can be defined as "diagnosis, treatment and/or prevention that complements mainstream medicine by contributing to a common whole, and by satisfying a demand either not met by orthodoxy or by diversifying the conceptual frameworks of medicine" [Ernst et al. 1995]. It has been argued that there is a prevailing orthodoxy with a skeptical and perhaps destructive attitude among the mainstream research community severely impeding the funding of research into alternative medicine [Ernst 1999]. Most published materials in this field are largely opinion based and rigorous research studies can be difficult to unearth in the literature. There are however over 450 published clinical trials investigating the efficacy of Chinese herbal medicine in a wide range of diseases including: asthma, dermatitis, eczema, cancer, diabetes mellitus, heart disease and irritable bowel syndrome, among others [Pach et al. 2002; Tang et al. 1999; Yuan and Lin 2000]. Many of these studies nevertheless are not easily accessible to a Western audience as the majority are written in Chinese or Japanese. To date, published work on the treatment of reproductive problems by Chinese herbal medicine mostly pertains to female infertility with fewer studies investigating the use of Chinese herbal medicine to improve sperm quality/quantity [Xu et al. 2003]. To the best of our knowledge, none have addressed the issue of whether there are relevant biochemical activities in the herbs commonly used for the treatment of male factor infertility. If alternative medicine is to be seriously considered a combination of double-blind placebo controlled clinical trials and assays that establish relevant biochemical activity demonstrating (*in-vitro* or *in-vivo*) the predicted effect in model systems, are essential. The purpose of the current study is to consider the second of these two approaches in the context of traditional Chinese herbal medicine (TCM) treatment for male infertility.

In the majority of cases, male factor infertility arises from defects in spermatogenesis. This can manifest itself either by a reduction in the number/quality of sperm or by the production of sperm that are compromised in their ability to fertilize an egg and/or promote normal healthy embryonic development. Spermatogenesis is a complex process under strict endocrine control that operates through a delicate balance and negative feedback mechanisms along the hypothalamic-pituitary-gonadal (HPG) axis. Any disruption/imbalance occurring within the HPG axis can thus result in infertility phenotypes including primary testicular failure, hypogonadotrophic hypogonadism, 5α-reductase deficiency, androgen insensitivity syndrome and partial androgen insensitivity syndrome, among others. The hypothalamus is required to secrete gonadotrophin-releasing hormone (GnRH) which acts on the anterior pituitary, in turn causing the release of both follicle stimulating hormone (FSH) and luteinizing hormone (LH). LH subsequently acts on the Leydig cells of the testes stimulating the production of testosterone by these cells; meanwhile FSH acts on the Sertoli cells stimulating the division of spermatogonia and activin and inhibin regulate negative feedback. It is beyond doubt that oestrogen is also fundamental to the maintenance of normal male fertility; mouse knockout models with inactivated oestrogen receptors result in diverse infertility phenotypes [Couse et al. 1999; Eddy et al. 1996; Krege et al. 1998; Robertson et al. 1999; Zhou et al. 2001]. It would appear, that oestrogen operates through negative feedback mechanisms [Santen 1975; Winters et al. 1979]. If a particular administered pharmacopoeia is to be effective, then it is a reasonable hypothesis that it may act via an endocrine pathway and thus possess endocrine activity.

Oxidative stress has also been implicated in male infertility with significant levels of reactive oxygen species (ROS) detected in 25% of infertile men, however fertile counterparts have no detectable levels of ROS [Iwasaki and Gagnon 1992]. Indeed significantly elevated generation of reactive oxygen species by abnormal spermatozoa and contaminating leukocytes (leukocytospermia) is one of very few defined male infertility aetiologies. The unsaturated fatty acids are targets for free radical attack and ongoing lipid peroxidation throughout the sperm plasma membrane resulting in the accumulation of lipid peroxides on the sperm surface causing sperm dysfunction and cell death [Aitken and Baker 2006; Sheweita et al. 2005]. Moreover, nuclear and mitochondrial DNA are subject to damage at low levels of oxidative stress; this in turn may lead to pregnancy loss [Aitken and Baker 2006; Lopes et al. 1998]. Evidence has been provided that semen samples from fertile men show an antioxidant capacity greater than their sub-fertile counterparts. In addition an inverse

correlation between antioxidant capacity and lipid peroxidation potential has also been identified, thereby suggesting that compromised antioxidant defenses could be involved in male infertility [Smith et al. 1996]. ROS-induced DNA damage may arise through the imbalance between endogenous or xenobiotic-induced ROS generation and the natural protection provided by the accessory sex glands [Leduc et al. 2008]. "Effective" Chinese herbs therefore might also act via an antioxidative mechanism and thus have antioxidant properties.

In the current study using a series of recombinant yeast based assays we tested the hypothesis that Chinese herbs typically used in the treatment of male infertility possess endocrine activity. These methods are popular screening systems due to their rapidity, cost-effectiveness and reliability in extrapolation to mammalian cell systems as nuclear receptors in yeast cells essentially behave as they do in mammalian cells [Breithofer et al. 1998; Coldham et al. 1997]. They are considered the "industry standard approach" to screen for endocrine activity and are capable of responding to oestrogens, xeno-oestrogens and androgens of either steroidal or non-steroidal origin. We also tested the hypothesis that these herbs possessed antioxidant activity using the ferric reducing antioxidant potential (FRAP) assay. The FRAP assay was chosen because of its reproducibility, cost-effectiveness, simplicity and speed [Benzie and Strain 1996]. It has been successfully applied in a number of studies investigating the antioxidant potential of plant homogenates, pharmacological plant products, herbal teas, medicinal plants [Szollosi and Varga 2002], common vegetables [Ou et al. 2002] and to investigate the presence of antioxidants in the follicular fluid of women undergoing *in-vitro* fertilization [Oyawoye et al. 2003].

RESULTS

Using the data generated in the endocrine assays, estimates of the oestrogenic, androgenic, anti-oestrogenic and anti-androgenic of the herb were directly compared with the known active standards of 17β-oestradiol, DHT, tamoxifen and flutamide (oestrogenic, androgenic, anti-oestrogenic and anti-androgenic, respectively). As illustrated in Figure 1, in each case the equivalent herb activity was calculated

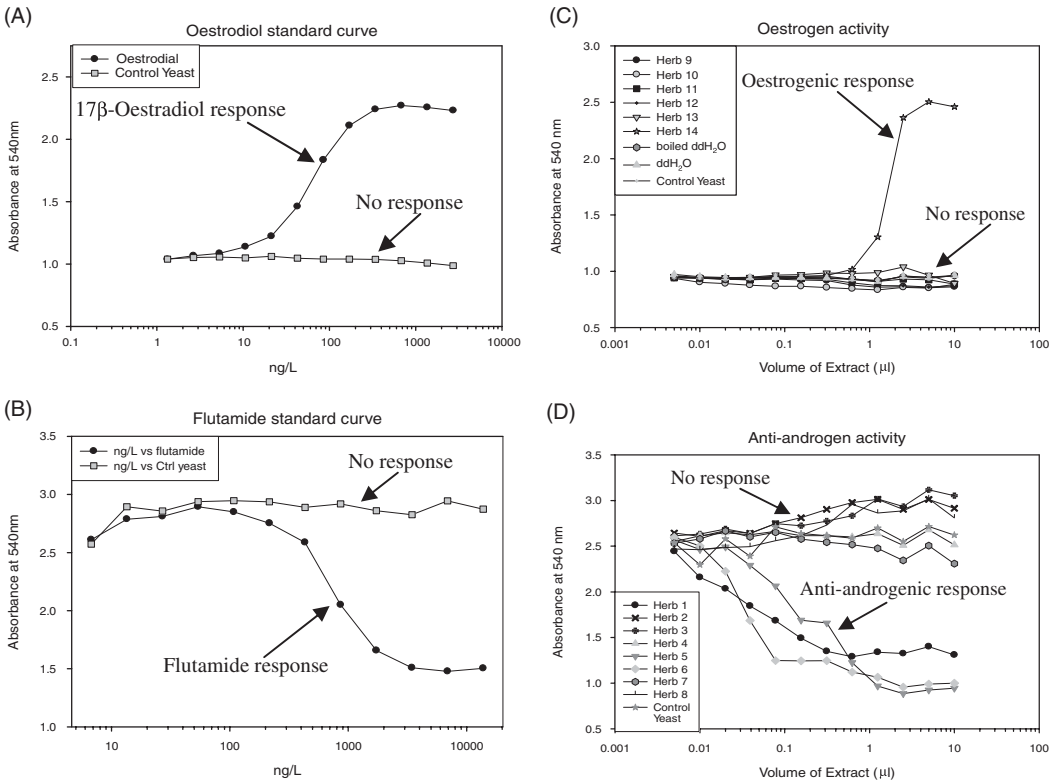

FIGURE 1 **Curves showing examples of standard (agonist and antagonist) responses. Examples of agonist and antagonist responses to 17β-oestradiol (A) and flutamide (B) and examples of individual herb responses for oestrogenic (C) and anti-androgenic (D) activity as shown.**

TABLE 1 **Activity and Property Summary.**

Herb number	Oestrogenic activity Oestradiol equivalents (ng/g)	Anti-oestrogenic activity Tamoxifen equivalents (mg/g)	Anti-androgenic activity Flutamide equivalents (mg/g)	Antioxidant activity % of ascorbic acid activity (100% is equivalent to 1.2 μg ascorbate equivalent, per mg dried herb)
Herb 1		**87.66** (± 12.1)	**25.52** (± 4.65)	**90%** (± 1.41)
Herb 2				33% (± 8.48)
Herb 3				0%
Herb 4		**7.68** (± 4.25)		60% (± 2.82)
Herb 5		**1280.66** (± 319.61)	**12.73** (± 1.43)	**105%** (± 2.82)
Herb 6		**78.51** (± 5.45)	**54.89** (± 7.63)	**91%** (± 4.24)
Herb 7		**2.58** (± 0.30)		23% (± 0.00)
Herb 8		**15.94** (± 4.36)		**84%** (± 7.07)
Herb 9		**8.77** (± 5.01)		18% (± 0.70)
Herb 10		**10.33** (± 1.88)	**66.78** (± 21.92)	**99%** (± 4.94)
Herb 11		**1.18** (± 0.71)		5% (± 7.07)
Herb 12		**7.39** (± 4.49)		**76%** (± 0.00)
Herb 13		0.98 (± 0.08)		27% (± 0.70)
Herb 14	**85.30** (± 14.14)			**100%** (± 2.82)
Herb 15		0.30 (± 0.21)		13% (± 4.24)
Herb 16		**2.12** (± 0.43)	0.32 (± 0.21)	38% (± 5.65)
Herb 17		0.06 (± 0.01)		24% (± 0.00)
Herb 18		0.52 (± 0.57)		39% (± 1.41)
Herb 19		**12.91** (± 5.60)	0.25 (± 0.02)	**99%** (± 9.89)
Herb 20	**550.00** (± 67.14)		0.17 (± 0.01)	56% (± 2.82)
Herb 21		**5.82** (± 0.62)		74% (± 7.07)
Herb 22		**27.05** (± 9.50)	**11.10** (± 1.98)	70% (± 8.48)
Herb 23		**4.63** (± 0.22)		**77%** (± 1.41)
Herb 24		**14.80** (± 0.52)		**98%** (± 5.65)
Herb 25				**89%** (± 5.65)
Herb 26				**91%** (± 0.00)
Herb 27		0.52 (± 0.57)		32% (± 0.00)
Herb 28		0.13 (± 0.19)		68% (± 4.94)
Herb 29		0.41 (± 0.01)	**4.52** (± 0.55)	24% (± 0.70)
Herb 30		0.93 (± 0.09)		**77%** (± 7.07)
Herb 31		**7.84** (± 1.37)	**1.54** (± 0.60)	27% (± 1.41)
Herb 32		**2.22** (± 0.54)		50% (± 0.00)
Herb 33		**16.26** (± 2.23)		**100%** (± 9.89)
Herb 34		0.17 (± 0.21)		59% (± 2.82)
Herb 35		**1.59** (± 0.02)		**100%** (± 0.70)
Herb 36		0.09 (± 0.00)		27% (± 4.24)
Herb 37				5% (± 0.70)
Ascorbic acid				100%
ddh_2O				0%
Decoction 1		0.22 (± 0.15)		**85%** (± 1.14)
Decoction 2		**1.14** (± 0.15)		**87%** (± 0.70)
Decoction 3		0.26 (± 0.01)		60% (± 4.24)
Decoction 4		**1.45** (± 0.62)		**82%** (± 0.00)
Decoction 5		**3.73** (± 0.38)		**100%** (± 8.48)
Decoction 6		**13.23** (± 0.72)		37% (± 0.70)
Decoction 7		**3.31** (± 0.01)		63% (± 2.82)

Oestrogenic, anti-oestrogenic, anti-androgenic activity (expressed as oestradiol, tamoxifen and flutamide equivalents/g) and antioxidant activity in individual herbs and decoctions. Herbs exhibiting strong endocrine and/or antioxidant activity are indicated in bold.

by plotting the activity of the standard expressed as ng/L and the volume of the herb extract within the experimental system as a function of absorbance measured at 540 nm. For each individual herb/herb decoction the absorbance at the mid response range along a linear section and the volume of herb extract was noted. Using the same absorbance on the dose response curve of the standard used in the assay

enabled an equivalent concentration of the herb compared to the standard to be calculated. Subsequently the "volume" of herb extract needed to elicit the response of a known amount of standard was calculated and activity in each extract was expressed as the amount (ng/mg) of standard needed to elicit the same response per gram of dried herb.

In the case of the antioxidant activity assessed from the FRAP assay, a standard curve was produced for the ascorbic acid and volume of herbal extract (1.25–40 µl). Antioxidant activity was calculated by comparing the activity of the herb extract (aqueous infusion of 150 g/l) with that of a 1 mM solution of ascorbic acid (expressed as a percentage). Thus "100%" is equivalent to 1.2 µg of ascorbate equivalent, per mg of dry weight herb.

All endocrine activity and antioxidant results are summarized in Table 1. Of the 37 individual herbs, two exhibited strong oestrogenic activity (ranging from 85.3–550 ng oestradiol equivalents/g dried herb); 20 demonstrated strong anti-oestrogenic activity (ranging from 1.18–1280.66 mg tamoxifen equivalents/g dried herb); 10 exhibited weak activity (ranging from 0.06–0.98 mg tamoxifen equivalents/g dried herb); seven exhibited strong anti-androgenic activity (ranging from 1.54–66.78 mg DHT equivalents/g dried herb); three exhibited weak anti-androgenic activity (ranging from 0.17–0.32 mg DHT equivalents/g dried herb) and no herbs displayed an androgenic response. Therefore the predominant endocrine activity of a significant proportion of the individual herbs was anti-oestrogenic. It is of note that the antagonist equivalents for the most part are significantly higher than for agonist assays and likely reflects that the assay does not consider the relative potencies of these compounds. For example 17β-oestradiol is approximately 10,000–15,000 fold more potent than tamoxifen.

Fifteen of the individual herbs exhibited strong antioxidant activity, defined as between 76 to greater than 100% of the activity of ascorbic acid (ranging from 0.91–1.26 µg acsorbate equivalent/mg dried herb), six herbs possessed intermediate levels (51–75%) (ranging from 0.6–0.88 µg ascorbate equivalent/mg dried herb), 16 herbs were found to have weak/no antioxidant activity (0–50%) (ranging from 0–0.46 µg acsorbate equivalent/mg dried herb).

The seven herb decoctions typically prescribed to patients undergoing male infertility treatment [Tempest et al. 2005] revealed that all seven only elicit an anti-oestrogenic response (Table 1); five strong anti-oestrogenic responses (ranging from 1.14–13.23 mg tamoxifen equivalents/g dried herb) and two weak anti-oestrogenic responses (ranging from 0.22–0.26 mg tamoxifen equivalents/g dried herb), with no significant response monitored for oestrogenic, androgenic or anti-androgenic assays consistent with their individual components. In addition, all seven decoctions exhibited antioxidant activity with three exhibiting strong antioxidant activity, three with intermediate activity and one showing low levels of activity (ranging from 1.02–1.2; 0.72–0.76 and 0.44 µg ascorbate equivalent/mg dried herb, respectively).

DISCUSSION

The reported success of traditional Chinese medicine (TCM) in the treatment of male infertility is still largely anecdotal and opinion-based. However several clinics report impressive success rates in their promotional literature [e.g. http://www.tcm-healthcare.co.uk/; http://www.cccm.co.uk/34.0.html]. Providing convincing evidence is far more difficult than for most other disorders since the successful outcome is ultimately measured by the conception of a healthy child [Agarwal et al. 2004]. Clearly this is complicated by the already low fecundity rate in humans and the fertility of the female partner. This study focused on a possible mechanism by which the therapeutic efficacious herbs might act. Confirmation of our initial hypothesis (that the herbs possess endocrine (specifically anti-oestrogenic and antioxidant activity) is consistent with relatively recent research suggesting that xenoestrogens and reactive oxidant species can cause male infertility.

Over the last decade substantial pharmacological research has been performed to investigate the chemical constituents of a variety of plant matter used in traditional Chinese medicine [Wang et al. 1995]. Most have revealed anti-thrombotic, anti-inflammatory, anti-allergic, anti-tussive and anti-bacterial effects [Gong and Sucher 1999].

To date there have been a handful of studies that have investigated 13 of the herbs tested in this study. Most have analyzed the antioxidant potential with a few investigating endocrine properties and responsiveness of these herbs. In the majority of cases these

herbs have been tested for their use in the treatment of diseases not related to male factor infertility. It should be noted that they are often not tested individually or are found in decoctions different to those described in this study. Thus it is difficult to draw direct comparisons between studies. Nevertheless even given the wide variety of both *in-vivo* and *in-vitro* assays, the results presented in this study are essentially in accord with those previously reported.

The most relevant of these studies in terms of male factor infertility will be considered first. Leydig TM3 mouse cells cultured with herb 15 (*Radix moridae officinalis*) have been shown to increase the production of testosterone having protective effects against hydrogen peroxide-induced stress [Chang et al. 2008]. It should be noted that the current study found herb 15 to have weak/no antioxidant activity. However, the amount of herb used is 5 fold lower in this study than that reporting an antioxidant effect. The effect of sperm motility and membrane function in human sperm after incubation with herb 12 (*Semen cuscutae*) and herb 15 has also been reported [Peng et al. 1997]. Incubation with herb 12 produced markedly improved motility and stabilized sperm membrane function, with herb 15 showing a relatively poor effect. It should be noted that in this study herb 12 possessed strong antioxidant activity compared to herb 15 with little/no activity (0.91 μg vs. 0.15 μg ascorbate equivalent per mg of dried herb). Antioxidant activity may play a role in stabilizing the sperm membrane and prevent/reduce lipid peroxidation of the sperm plasma membrane that may result in sperm dysfunction and cell death [Aitken and Baker 2006; Sheweita et al. 2005]. Flavonoids extracted from herb 12 were administered in immature rats through gastric gavage to observe the effects on the reproductive system [Qin et al. 2000]. The results from this study suggest that flavonoids extracted from herb 12 can stimulate the reproductive system and endocrine function in rats as was observed through the increase in weight of the testis, epididymis and pituitary gland. In addition, secretion of testosterone and luteinizing hormone (LH) was stimulated in both *in-vitro* and immature rats. Interestingly, herb 12 elicited a strong anti-oestrogenic activity but had no measurable androgenic activity. Oestrogen clearly has a role in the male reproductive endocrine pathway as oestrogen receptor mouse knock out models display a range of infertility phenotypes. Its role is not yet clear, however, it is believed to operate through negative feedback mechanisms. Perhaps, the use of such herbs may counteract excess oestrogen found in the body through diet/environment or through altering the negative feedback mechanisms resulting in the increased production of testosterone and LH as seen in this study. Another alternative to consider is that the flavonoids extracted may possess different activities to what herb 12 would elicit.

The oestrogenic activity of herb 30 (*Rhizoma cimicifugae*) has previously been tested as it is often used effectively in the treatment of peri- and post-menopausal symptoms. However the results suggest that this plant does not possess agonist activity but rather seems to act as a weak anti-oestrogen [Kretzschmar et al. 2005]. The effects on hormone responsive and hormone-refractory prostate carcinoma cells of a decoction of nine herbs including herbs 7 (*Fructus ligustri lucidi*) and 15 (*Radix moridae officinalis*) have previously been tested. The results suggest that the decoction tested may be efficacious in preventing or treating androgen independent prostate carcinoma through reduced cancer cell growth, induced apoptosis and suppressed expression of the androgen receptor [Hsieh et al. 2002].

This study has confirmed previous reports of the antioxidant activity of 12 herbs including herbs: 5-*Cortex phellodendri*; 7-*Fructus ligustri lucidi* (extracted glucosides); 9-*Rhizoma dioscoreae*; 10-*Fructus corni*; 12-*Semen cuscutae*; 13-*Rhizoma alismatis*; 15-*Radix moridae officinalis*; 19-*Fructus rubi*; 21-*Flos carthami tinctorii*; 28-*Rhizoma atractylodis*; 31-*Radix astragali seuhedysari*; 35-*Flos lonicerae japonicae*) [Chang et al. 2008; Kong et al. 2001; Koo et al. 2004; Li et al. 2007; Liu et al. 2003; Satoh et al. 2004; Xuejiang et al. 2001]. Several herbs have been shown to be capable of strong hydroxyl (*OH) scavenging activity (herbs 5; 21; 28; 31) [Kong et al. 2001; Satoh et al. 2004; Xuejiang et al. 2001]. Herbs 28 and 31 in decoction (containing two herbs not used in this study), was capable of preventing cerebral oxidative injury in rats *in-vivo* [Xuejiang et al. 2001]. In addition, various preparations of herb 5 (the strongest antioxidant in this study, 1.26 μg ascorbate equivalent/mg dried herb) were also found to be capable of scavenging superoxide radicals and in addition inhibit lipid peroxidation [Kong et al. 2001]. Dietary intake/*in-vitro* incubation of decoctions

including herb 21 in senesce accelerated mice [Satoh et al. 2004] and herbs 9, 12 and 35 in D-galactose-induced mimetic aging mice [Liu et al. 2003] and herbs 9, 10, 13, 15 and 19 in the rat pheochromocytoma line PC12 [Koo et al. 2004] have demonstrated enhanced activities of antioxidant enzymes including: superoxide dismutase, catalase and glutathione peroxidase indicating cytoprotective effects against reactive oxidants by improving antioxidant status.

The work of Sumpter and colleagues [Sumpter 1998; Sumpter and Johnson 2005; Tyler et al. 1998] has established that minute quantities of xenoestrogens in the environment can cause sex reversal and infertility in fish. This has inevitably led to extrapolation of the possible effects on human fertility in light of the reported reduction in sperm counts over the last 100 years [Sharpe 1993]. In addition, a potential association between environmental and genetic factors may result in testicular dysgenesis syndrome (TDS) [Skakkebaek et al. 2001]. The mechanism by which TDS arises remains to be elucidated, however, *in-utero* exposure to endocrine disrupters in particular oestrogens and anti-androgens in animal models have resulted in similar TDS phenotypes as seen in humans [Fisher et al. 2003].

Evidence of whether intake of antioxidants improves male fertility is contentious with mixed results in part due to antioxidants being used in different combinations and dosages for varying durations and for different pathologies [Agarwal et al. 2004]. It would seem judicious, to monitor "oxidative stress status" in individual men to enable the design/identification of an effective antioxidant treatment regime that may ultimately be driven by the source of the oxidative stress. Implementing this strategy should enable underlying imbalances to be corrected thereby encouraging normal spermatogenesis in males identified with high levels of ROS [Sheweita et al. 2005; Twigg et al. 1998].

To the best of our knowledge this is the first study to investigate endocrine and antioxidant activity in Chinese therapeutic herbs prescribed in the treatment of human male infertility. It should be stressed however, that although we have determined that these herbs possess measurable and quantifiable biological activities, the results should be regarded as preliminary as the *in-vivo* mechanisms by which they may be acting remain the subject of future studies. It is conceivable that these complex decoctions may be capable of targeting and interacting with multiple signal transduction/endocrine/oxidant/metabolic pathways to effectively correct, counteract or circumvent the impaired or dysfunctional mechanisms of various male factor infertility phenotypes.

The integration of traditional Chinese and Western pharmacology potentially constitutes a rich source for drug discovery and development [Gong and Sucher, 1999], however, the analysis and standardization of TCM formulae (the majority of which are a complex mixture of any number of individual components) is not a simple task. Isolating active compounds is similarly complex and any therapeutic efficacy of traditional Chinese medicine may be related to the pharmacokinetic or pharmodynamic synergism of the ingredients with potentially many different effects contributing to the overall response. Moreover any potential effects may be the result of the metabolism of these compounds, the product of which may be responsible for the resultant activities. Therefore a multitude of tests must be undertaken to investigate the effects of such complex preparations. Of further concern are considerations pertaining to the chemical constituents of the herbs that must ultimately be identified and standardized if the treatment is to enter the mainstream of Western practice. Herbs harvested at different times of year and within different geographical regions, climates, altitude and seasons may possess varying chemical properties [Drew and Myers 1997]. Contamination is an issue with residues of pesticides, heavy metals and toxic elements as well as unwanted plant constituents such as weeds and foreign matter, as is the safety and efficacy of the treatment since the majority of herbal products are unlicensed in most countries.

Unbiased scientific evaluation will bring about a greater understanding of the efficacy (or otherwise) of therapeutic herbs and their validation as an accepted form of treatment. *In-vitro* assays, such as those undertaken in this study can be used to take significant steps towards making this a reality.

MATERIALS AND METHODS

Traditional Chinese herbs were obtained from "Beijing Tong Ren Tang" (Shaftesbury Avenue, London, UK).

TABLE 2 **Latin, Pin-Yin, and Common Names of Each of the 37 Individual Herbs.**

Herb no.	Latin name	Pin Yin name	Common name
Herb 1	*Radix paeoniae rubrae*	Chi Shao	Red peony root
Herb 2	*Radix cyathula officinalis*	Chuan Niu Xi	Cyathula root
Herb 3	*Sclerotium poriae cocos*	Fu Ling	Poria
Herb 4	*Fructus lycii chinensis*	Gou Qi Zi	Lycium fruit
Herb 5	*Cortex phellodendri*	Huang Bi	Phellodrendron bark
Herb 6	*Cortex moudan radicis*	Mu Dan Pi	Moutan
Herb 7	*Fructus ligustri lucidi*	Nu Zhen Zi	Ligustrum seed/glossy privet fruit
Herb 8	*Rehmannia glutinosa Libosch*	Shu Di	Oriental or Korean foxglove
Herb 9	*Rhizoma dioscoreae*	Shan Yao	Chinese yam
Herb 10	*Fructus corni officinalis*	Shan Zhu Yu	Cornus fruit/dogwood fruit
Herb 11	*Semen persicae*	Tao Ren	Persica
Herb 12	*Semen cuscutae*	Tu Si Zi	Dadder seed/ cuscuta seed
Herb 13	*Rhizoma alismatis plantago-aquaticae*	Ze Xie	Alismatis rhizome/ water plantain tuber
Herb 14	*Rhizoma anemarrhenae asphodeloidis*	Zhi Mu	Anemarrhena
Herb 15	*Radix moridae officinalis*	Ba Ji Tian	Morinda root
Herb 16	*Rhizoma atractylodis macrocephalae*	Bai Zhu	Alba atractylodes
Herb 17	*Semen plantaginis*	Che Qian Zi	Plantago seed
Herb 18	*Radix codonopsis pilosulae*	Dang Shen	Codonopsis
Herb 19	*Fructus rubi*	Fu Pen Zi	Rubus
Herb 20	*Radix glycyrrhizae uralensis*	Gan Cao	Liquorice root
Herb 21	*Flos carthami tinctorii*	Hong Hua	Safflower flower
Herb 22	*Semen varricariae pyramidata medic*	Wang Bu Liu Xing	Vaccaria seed
Herb 23	*Fructus schisandrae chinensis*	Wu Wei Zi	Schisandra fruit
Herb 24	*Aurantii fructus*	Zhi Ke	Aurantium fruit
Herb 25	*Acori graminei rhizome*	Shi Chang Pu	Acorus rhizome
Herb 26	*Nelumbinis embryo*	Lian Zi Xin	Lotus embryo
Herb 27	*Rhizoma dioscoreae septemlobae*	Bi Xie	Hypoglauca yam
Herb 28	*Rhizoma atractylodis*	Cang Zhu	Attractylodes
Herb 29	*Radix bupleuri chinensis*	Chai Hu	Bupleurum
Herb 30	*Rhizoma cimicifugae*	Sheng Ma	Bugbane rhizome
Herb 31	*Radix astragali seuhedysari*	Huang Qi	Astragalus root
Herb 32	*Radix rehmanniae glutinosae*	Sheng Di Huang	Fresh rehmannia
Herb 33	*Pericarpium citri reticulata blanco*	Chen Pi	Citrus peel
Herb 34	*Eclipta prostrata*	Han Lian Cao	Eclipta
Herb 35	*Flos lonicerae japonicae*	Jin Yin Hua	Lonicera flower
Herb 36	Herba epimedii	Yin Yang Huo	Epimedium
Herb 37	*Semen coicis*	Yi Yi Ren	Coix seed/jobs tear seed

Preparation of Herbal Extracts

The herbal extracts were prepared as follows: 37 individual plant-based herbs (Table 2) used in the treatment of male infertility by TCM and seven herb "decoctions" prescribed to the male patients were tested. Each decoction was a formula previously used on a patient for treatment of male infertility.

- Decoction 1 consisted of herbs 2–4, 6–11, 21 and 23 (9.7, 9.7, 9.7, 7.3, 9.7, 21.1, 9.7, 8.1, 7.3, 9.7 and 7.3% by mass, respectively).
- Decoction 2 consisted of herbs 3–6, 8–14 and 21 (9.2, 9.2, 9.2, 7.6, 11.5, 9.2, 7.6, 6.9, 9.2, 7.6, 9.2 and 3.8% by mass, respectively).
- Decoction 3 consisted of herbs 7, 16, 18, 20, 29–31, 33 and 34 (13.8, 10.3, 17.2, 6.9, 6.9, 6.9, 17.2, 6.9 and 13.8% by mass, respectively).
- Decoction 4 consisted of herbs 1, 3, 5–7, 9, 10, 14, 24, 32, 34, and 35 (4.6, 9.1, 9.1, 7.6, 9.1, 9.1, 7.6, 9.1, 7.6, 11.4, 9.1, 6.8% by mass, respectively).
- Decoction 5 consisted of herbs 3, 6, 8–10, 12, 13, 17, 19, 23 and 24 (9.5, 7.9, 11.9, 11.9, 7.9, 9.5, 7.1, 9.5, 7.1, 9.5, 7.1 and 7.4% by mass, respectively).
- Decoction 6 consisted of herbs 4, 12, 16, 18, 20, 30, 31 and 33 (16.7, 16.7, 13.3, 16.7, 6.7, 6.7, 16.7 and 6.7% by mass, respectively).
- Decoction 7 consisted of herbs 3, 5, 17, 18, 25–28 and 36 (11.5, 11.5, 9.6, 14.4, 9.6, 11.5, 14.4, 11.5 and 5.8% by mass, respectively).

The recommended preparation of the herbs was adapted for laboratory use (scaled down), while abiding to the preparation guidelines set for patients. In brief, 0.15 g of each investigated herb was placed into a sterile Pyrex tube to which 1 ml of double distilled water was added; this was left to infuse overnight (room temperature) and subsequently boiled for 30 min.

Endocrine Assays

In order to establish if any herbs possessed endocrine activity an established recombinant yeast screen was used [Routledge and Sumpter 1996; Sohoni and Sumpter 1998]. Boiled aqueous herb extracts were serially diluted (1:10) and air dried before being assayed for endocrine activity. In brief, the recombinant yeast cells have a genetically modified genome containing either a human oestrogen receptor gene (hER) or a human androgen receptor gene (hAR). The assay tests compounds that have the ability to interact with the human oestrogen/androgen receptor. An active ligand occupies the steroid hormone receptor, causing it to bind to the corresponding DNA response element initiating transcription of the *lac-Z* reporter gene. The β-galactosidase substrate—CPRG (chlorophenol red-B-D-galactopyranoside) is then metabolized and product measured at 540 nm [Routledge and Sumpter 1996]. It is also possible to detect antagonist activity as well as agonist activity through the addition of a sub-maximal concentration of an agonist to the medium as in the case of the 17β-oestradiol and dihydrotestosterone (DHT) anti-androgen assay. In the presence of an antagonist the activity of the agonist will be inhibited in a dose-dependent fashion. Hence the antagonist will compete for the receptor and inhibit the color change [Routledge and Sumpter 1996; Sohoni and Sumpter 1998]. Detailed assay procedures and the preparation of yeast growth medium has previously been reported [Routledge and Sumpter 1996; Sohoni and Sumpter 1998].

Assay plates were analyzed at 540 nm to assess activity and 620 nm to assess yeast growth. Colorimetric corrected value = chemical absorbance (540 nm)-[chemical absorbance (620 nm)-blank absorbance (620 nm)].

All recombinant yeast screens positive controls (standards) were at the following concentrations: oestrogen screen–17β-oestradiol (1.3–2,724 ng/l), androgen screen–dihydrotestosterone (DHT, 7–14,520 ng/l), anti-oestrogen–tamoxifen (9.4–19,375 μg/l) and anti-androgen–flutamide (7–13,810 μg/l). Negative controls were included to assess contamination within the assay; these included a yeast media and ddH_2O used to dilute the herbal extracts.

Antioxidant Assay

The ferric reducing antioxidant potential (FRAP) assay was used to determine the antioxidant potential of the herb and decoction infusions. FRAP employs the principle that the presence of an antioxidant results in ferric to ferrous ion reduction at a low pH resulting in the formation of a colored ferrous-tripyridyltriazine complex. The level of FRAP is determined at 593 nm in comparison to that of a known concentration of ferrous ions [Benzie and Strain 1996].

The working FRAP reagent was produced by combining in a 10:1:1 (volume to volume) ratio, respectively, 300 mM acetate, pH 3.6, buffer; 10 mM 2,4,6-tripyridyl-*s*-triazine in 40 mM HCl and 20 mM $FeCl_3 6H_2O$. FRAP reagent (1 mL) was added to 1.25, 2.5, 5, 10, 20 and 40 μl of the boiled aqueous herbal extract (prepared as above) and subsequently incubated in the dark at room temperature for 10 min. Immediately following this incubation absorption at 593 nm was determined. A solution of 1 mM ascorbic acid provided a positive control while ddH_2O was used as the negative control.

All the individual herbs/herb decoctions were assayed for endocrine and antioxidant activity on at least two separate occasions with a small standard deviation.

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