American Herbal Pharmacopoeia®
and Therapeutic Compendium

Red Clover Flowering Tops, Aerial Parts, and Dry Extracts

Trifolium pratense L.

Standards of Identity, Analysis, and Quality Control

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Trifolium pratense
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NOMENCLATURE

Botanical Nomenclature

Trifolium pratense L.

Botanical Family

Fabaceae (Leguminosae)

Pharmacopoeial Name

Trifolii flos, Trifolii herba

Pharmacopoeial Definition

Red clover consists of the flowering tops and/or aerial parts of *Trifolium pratense* L. containing not less 0.5% total isoflavones calculated on a dry weight basis as the sum of biochanin A, daidzein, formononetin, and genistein.

Common Names

English:	Red clover
European Union:	Red clover
French:	Trèfle rouge; trèfle des prés
German:	Rotklee, Rotklee blüten
Spanish:	Trébol rojo

HISTORY

Nomenclature and a New World Introduction

There is not a lot of historical data chronicling the medical use of red clover *Trifolium pratense*. In many cultures, various species of clover have been used as food, fodder, and medicine. The genus name *Trifolium comes* from the Greek *triphullon*, referring to the trifoliate (three-parted) palmate leaves, which occur in groups of three and are characteristic of about 230 species within the genus *Trifolium* (Hyam and Pankhurst 1995). The species name *pratense* is derived from the Latin *pratensis*, meaning "of meadows" (Gledhill 1989), so indicative of the common habitat of red clover. Thus, *T. pratense* is a plant with trifoliate leaves typically found in meadows. Many of the *Trifolium* plants are most familiar as clovers; this species is commonly known as red clover due to its purple red flowers, which also possess a sweet fragrance, making them attractive to honey bees and providing a resource for clover honey. Early authors refer to this species as cultivated clover (e.g., Afzelius 1791).

Red clover is native to Europe (Fernald 1950) and is widely distributed throughout North America. Though it is unknown when the plant was introduced into North America, Pieters (1926), an agronomy specialist in clovers, reported that it has been used as a cover crop in the US for at least 200 years, dating it to approximately 1726, and describing it as "a corner stone of a permanent system of agriculture in the Old World" and "a leading factor in conserving productivity in the soil." in North America. In a work on agricultural plants by Jared Eliot (1747 in Pieters 1926), red clover was reported to have already been adapted to New England by the middle 1700s.

Record of Red Clover Use in European and English Herbals

Reference to red clover is lacking in many of the earliest most seminal works of botanical medicine, including that of Dioscorides (1st century CE), which remained the authoritative text of medicine for several centuries.

One of the earliest records of the medicinal use of red clover is from the German Abyss, Hildegard von Bingen (1098–1179), who, in her *Physica*, recommends the use of the herb for clearing obscured vision by macerating flowers in oil and anointing the eyes and eyelids (Throop 1998). As many of Hildegard's herbal medicine recommendations have their origin in earlier historical literature (e.g., Dioscorides), it is likely that her use of red clover was already established in the earlier common medical literature. *Trifolium* was included in *Hortus Sanitatus*, the earliest of German encyclopedias of natural history in 1497 (Anon 1497; first published in 1485). One of the earliest of the seminal European herbals reporting on the medicinal use of red clover is *De Historia Stirpium Commentarii Insignes* of Leonhart Fuchs (1542), in which Fuchs reports the herb's benefit in treating white discharges (leucorrhea) in women and topically to promote the suppuration of growths and boils. Fuchs also noted that red clover was not in widespread use by apothecaries of the time, predominantly because they were looking for good tasting preparations to sell. This was followed by the *New Herball* of William Turner (1568) that recommended red clover leaves with and without flowers for the treatment of pleurisy, epilepsy, fevers, and poisonous bites and as a diuretic. *The Herball* of John Gerard (1597) reports on the local or topical use of red clover as a rectal injection for "stomach paines and poultice for swellings and inflammations". Interestingly, most of the medicinal applications of these herbals differ from each other and those of Hildegard's *Physica* suggesting indistinct and varied origins that for now remain unknown.

Another herbal writer of the Renaissance period, Mattioli (1626), while an authority on the writings of Dioscorides, was unique among the early herbal classic writers in providing commentary on folk uses of botanicals. Of red clover Mattioli wrote that the flowers with the seeds boiled in honey water or wine and drunk or used as an enema (clyster) will soften hard and stringy phlegm in the bowels and heal the wounds and the pains. Mattioli also reported; "people say if you eat clover of the purplish-brownish flowers or drink them it can make menses irregular. But the one with the white flowers makes it flow."

Red clover also appears in *Theatrum Botanicum* (1640) of the noted apothecary John Parkinson, who recorded that the juice was used topically to treat venomous snakebites and as an ophthalmic preparation "to clear the eyes of any film beginning to grow over them, or to soothe them when hot and bloodshot." In 1696, the prolific medical writer William Salmon in his *Pharmacopoeia Londinensis*

reported on a wide range of actions and indications that are broader than most provided by other writers (see Traditional Western Herbal Medicine Supplement). Salmon in his *Botanologia* of 1710 provided more detailed prescriptive information. The noted Swedish botanist, Carolus Linnaeus, in his *Materia Medica* (1749), recorded the herb's singular use for arthritis.

Red clover does not appear to figure prominently in the 18th to 20th century healing traditions of England, France, Germany, or Switzerland as the herb is lacking in numerous representative works of these traditions (e.g., Artus 1887 [Germany]; Guibourt 1831 [France]; Hill 1812 [England]; Lust 1959 [Germany]; Quincy 1762 [England]; Thornton 1810 [England]; Vogel 1952 [Switzerland]. Red clover reemerges in more modern folk traditions.

Figure 1 Historical illustration of red clover

Source: Fuchs L. 1542. De *Historia Stirpium Commentarii Insignes* Aboca reprint. Courtesy of Aboca, Sansepolcro, Italy.

In the British Isles, red clover tea was used to treat coughs, colds, and cancer, and it was used as a topical preparation for bee stings, rashes, and toothaches (Allen and Hatfield 2004). In Ireland, red clover was reportedly powdered and mixed with bread as a food crop when other foods were scarce (Hedrick 1919).

Use of Red Clover by Native Americans

Despite the relatively new introduction of red clover to the New World, red clover has a widespread tradition of medicinal use among Native Americans. An infusion was taken by Quebec First Nations peoples for whooping cough (Moerman 2013), the Cherokee used the plant as a febrifuge, Bright's disease, and leucorrhea (Hamel and Chiltoskey 1975), the Iroquois (blossoms) and Rappahannock (leaves and stems) used the plant as a "blood medicine," and the Shinnecocks (decoction of powder) and Thompson (infusion of blossoms), respectively, used red clover to treat cancer in general, and stomach cancer specifically (Moerman 2013; Turner et al. 1990). The Iroquois reportedly used a cold infusion of red clover blossoms for 'change of life', providing the earliest historical support for the now popular use of red clover (e.g., Promensil®, Novogen Ltd, Australia) for menopause (Herrick 1995). In addition to its medicinal use, red clover was used as a food crop. Tribes in the Central region of California cooked clover by placing layers of the well moistened plant between hot stones and reportedly consumed large quantities (Hedrick 1919). The Apaches of Arizona included red clover among other greens and boiled them.

Use of Red Clover in the American Herbal Literature

John Bartram (1699–1777), a noted early American medical botanist, recorded seeing red clover growing in North America prior to the American Revolution (1765), while a Samuel Deane in 1797 recorded that the plant was highly valued in New England (both reported by Hedrick 1919). By the mid-18th century, red clover may have still been used in folk medicine but it seems to be neglected in most of the later 18th century and early 19th century herbals. Samuel Stearns in his *American Herbal* (1801), the first formal herbal printed in America, did note that some Americans drank the blossoms as tea instead of bohea tea (Chinese black tea), but otherwise reported the herb's predominant use as feed for cattle, sheep, and horses. Red clover's reintroduction to western usage appears to be due to Samuel Thomson (1835), the founder of the first American system of botanic medicine, Thomsonian medicine. Thompson used the herb as a "cancer plaster". Homeopaths and Eclectics also either made mention of (Millspaugh 1887) or recognized the medicinal use of red clover (e.g., Beach 1859; Felter and Lloyd 1909). Michael Moore (2003) astutely notes that at this time, the term cancer referred to "any necrotic sore that healed slowly and derived from below the surface" and did not necessarily coincide with a confirmed diagnosis of cancer today. The following references to the use of red clover in cancer should consider this caveat.

John King and Robert Newton (1852) provide one of the earliest reports on the eclectic use of red clover, stating that a decoction of the blossoms should be cooked down to the "consistence of an extract". The extract was applied topically by spreading on linen or thin leather to treat cancerous ulcers and bad burns. Wooster Beach, considered by many

Table 1 Historical timeline on the use of red clover (*Trifolium pratense*)

1098–1179 CE	The German Abyss, Hildegard von Binge recommends the use of red clover for clearing obscured vision by macerating flowers in oil and anointing the eyes and eyelids.
1497	Briefly cited in the *Hortus Sanitatus*, the earliest of German encyclopedias of natural history.
1542	Leonhardt Fuchs in his *De Historia Stirpium Commentarii Insignes* records the herb's benefit in treating white discharges (leucorrhea) in women and topically to promote the suppuration of growths and boils. Fuchs notes that red clover was not in widespread use by apothecaries of the time.
1568	William Turner in his *New Herball* recommend the use of red clover leaves with and without the flowers for the treatment of pleurisy, epilepsy, fevers, poisonous bites, and as a diuretic.
1597	John Gerard in his *The Herball* reports the local or topical use of red clover as a rectal injection for "stomach paines" and as a poultice for "swellings and inflammations."
1640	John Parkinson, in his *Theatrum Botanicum*, states the juice of red clover was used topically to treat venomous snakebites and as an ophthalmic preparation "to clear the eyes of any film beginning to grow over them, or to soothe them when hot and bloodshot".
1749	Noted Swedish botanist Carolus Linnaeus reports in his *Materia Medica* on the herb's singular use for arthritis.
1801	In Samuel Stearn's *American Herbal*, the first formal herbal published in America, red clover is mentioned as being drunk as an alternative to Chinese black tea.
Native American Use	Various First Nations peoples of North America utilize red clover blossom tea (infusion and decoction) for whooping cough, as a "blood medicine", and for "cancer".
1833	Samuel Thomson, the founder of the Thomsonian system of medicine popularizes the use of red clover with other herbs as part of a "cancer plaster".
1834	Wooster Beach, founder of the Reformed practice of medicine (Eclectics), includes red clover in his *The British and American Reformed Practice of Medicine* citing the herb's use as an emollient and antiseptic very useful for old sores, scrofulous tumors, and sore lips. Beach reports that red clover, used internally, possesses "valuable detergent properties in diseases of the blood" and to allay stomach acidity, although questions its efficacy in cancer.
1890	Red clover blossoms are included in the *Organic Materia Medica* published by the Parke Davis and Company citing: deobstruent and sedative actions, the herbs use in whooping cough, scrofula, and as an external wash for ulcers. This reference also reports that Dr Benjamin Rush, a Revolutionary war hero, one of four physician signers of the Declaration of Independence, and one of the most noted physicians of the time, considered *Trifolium Compound Syrup* to be the most superior of alteratives used for the treatment of syphilis.
1910–1942	Red clover blossoms are included in the 20th–21st and 4th–7th editions of the *United States Dispensatory* (USD) and *National Formulary*, respectively, the USDs noting there being a lack of evidence regarding the physiological activity of the plant.
1924	Harry Hoxsey introduces the now famous *Hoxsey Formula* (a variation of the Eclectic *Trifolium Compound*) as a purported cure for cancer, popularizing the herb's use for cancer and incurring the wrath of federal regulators.
1939	Jethro Kloss in his *Back to Eden* records red clover blossoms as possessing depurative (blood purifying), detergent, alterative, and mild stimulant effects and reports on the high regard placed on the herb by Seventh Day Adventist founder Ellen G White, calling it one of God's "greatest gifts to man."
1992-1996	Red clover flower is included in the *British Herbal Compendium* (Bradley 1992) and *British Herbal Pharmacopoeia* citing the herb's use as a "dermatological agent" and possessing "mildly antispasmodic, expectorant," and "anti-inflammatory" activity.
1997	A dietary supplement containing a red clover extract is launched in Australia, leading to the popular use of red clover for the relief of menopausal symptoms.
2003	Red clover blossoms entered into the *United States Pharmacopeia* (USP26)-*National Formulary* (USP26-NF21).
2013–2015	The *United States Pharmacopeia* revises red clover monographs to include inflorescence and aerial parts and in 2015, propose a monograph for "Red Clover Aerial Parts Isoflavones Aglycone Dry Extract".

as the founder of the Reformed practice of medicine, which transformed into the Eclectic medical movement, included red clover in his book *The British and American Reformed Practice of Medicine* (1859). Beach recommended the external use of the blossoms as an emollient and antiseptic that was very useful for old sores, scrofulous tumors, and sore lips and suggested that, used internally, the herb possessed "valuable detergent properties in diseases of the blood" and could allay stomach acidity. Beach, however, questioned the efficacy of the herb for cancer.

Among the Eclectics red clover was known as an effective alterative, a remedy considered to alter morbid conditions, and as a remedy for pertussis, "spasmodic coughs, as those of measles, bronchitis, laryngitis, phthisis, etc." and for "individuals disposed to tibial and other forms of ulcers", as well as an agent retarding the growth of certain cancers (Felter and Lloyd 1909). Because of red clover's many uses, various preparations known as "trifolium compounds" were manufactured by eclectic firms such as William S Merrill chemical company and conventional pharmaceutical companies such as Parke Davis & Co, among others (Tyler 1993).

Millspaugh, in his *American Medicinal Plants* (1887), only gave a cursory mention of a tincture of red clover made from the fresh blossoms reporting on the actions of a Dr TC Duncan who simply recorded the traditional indications of earlier herbalists noting the specificity of the botanical for dry, irritated throats, hacking coughs, and constipation.

Red clover was also included in the *Organic Materia Medica* of the noted pharmaceutical manufacturer Parke Davis & Company (Davis 1890) in which deobstruent and sedative actions were recorded, also noting its use for whooping cough, scrofula, and as an external wash for ulcers. This reference also reports that Dr Benjamin Rush, a Revolutionary war hero, one of four physician signers of the Declaration of Independence, and one of the most noted physicians of the time, considered *Trifolium Compound Syrup* to be the most superior of alteratives used for the treatment of syphilis.

Red Clover in Official Historic US Compendium

Red clover was subjected to extensive chemical analysis in 1910 (Power and Salway 1910), and was included in the 20th–25th editions of the *United States Dispensatory* (USD), due more to its popularity as a remedial agent than any demonstrable research findings. The USD reported on the historical use of red clover, mostly of the Eclectics, noting that at one time, "attributed with alterative and antispasmodic properties and used in whooping cough and scrofula. It has also been used in the form of ointments and local applications to ulcers. It also enters into the composition of some "antiasthmatic cigarettes." While reporting on these historic uses, both sources disparaged the plant's use, the USD reporting the herb as having "no sufficient reason to suspect it of any medicinal virtue." The USD also reported that subsequent chemical analysis by Salway in 1913 and Nakaoki in 1935 identified several glycosides, but concluded, "There is no evidence that any of these principles is physiologically active" (Osol and Farrar 1955). Red clover blossoms and its preparations were similarly included in the 4th edition of the *National Formulary* (National Formulary 1916), a status maintained until the 7th edition (National Formulary 1942).

Modern Use of Red Clover by American Herbalists

Red clover features prominently in the modern naturopathic and herbal literature, being included in the works of the Dominion Herbal College (Nowell 1926), Kuts-Cheraux's *Naturae Medicina* (1953), and *Herbal Medications* of Priest and Priest (1982). The uses most commonly reported in this literature include treatment of spasmodic coughs and for its alterative properties, predominantly as a tea or fluidextract. In his *Back to Eden*, 7th Day Adventist Jethro Kloss (1939) reports on the fondness of red clover by Adventist founder E White, regarding it as "one of God's greatest gifts to man."

Red clover, as noted, did not receive official status in early editions of the *United States Pharmacopeia* (USP), but is listed in the 1992 edition of the *British Herbal Compendium* and the 1996 edition of the *British Herbal Pharmacopoeia*. These British works cite the following uses of red clover: "dermatological agent", "mildly antispasmodic, expectorant," and "anti-inflammatory", despite a lack of supporting pharmacological data (Bradley 1992; *British Herbal Pharmacopoeia* 1996). Red clover flowering tops were entered into the *USP-National Formulary* (USP-NF 22) in 2004, and, in 2013, was revised to include in red clover aerial parts. Red clover appears to not be widely used in either Ayurvedic or traditional Chinese medicine.

Red clover is not official in any of the other major pharmacopoeias (e.g., *European Pharmacopoeia*, *Pharmacopoeia of the People's Republic of China*, among others. Despite this lack of official recognition, red clover continues to be used as a common ingredient in herbal compounds throughout the United States and Europe. One of its most enduring legacies is its purported benefit in treating cancer. It is an ingredient in the famous Hoxsey cancer formula announced in 1924 by Harry Hoxsey, a treatment claimed to be based upon Hoxsey's great grandfather's observation that a cancerous horse "cured" itself by feeding on certain plants. However, this story is most assuredly fabricated. The Hoxsey formula, along with many similar variations, is almost identical to the well-known previously mentioned Eclectic preparation *Trifolium Compound* also known as *Fluidextractum Trifolii Compositum*. Additionally, it would be an almost impossibility to have all the herbs growing in a single meadow and horses do not generally dig roots, calling into question the claim of Hoxsey. Despite marketing exaggerations, the reputation of red clover as an anticancer agent is long standing and persistent in the literature (Hartwell 1982). Some support for anticancer activity is evidence from pre-clinical studies demonstrating that the isoflavonoid biochanin A (a primary constituent of *T. pratense*) has an inhibitory effect on carcinogenesis (Buckingham et al.1994; COCTONOC-NRC 1996; Moon et al. 2008; Szliszka et al. 2013).

Most recently, attention has been focused on the fact that red clover is rich in isoflavones, which has resulted in its use as a "phytoestrogen", being especially used in the relief of menopausal symptoms, but also has been investigated for its potential benefits in cardiovascular disease,

diabetes, and metabolic syndrome. Numerous species of clover including *T. alpestre*, *T. baccarini*, *T. tembense*, *T. globosum*, *T. israeliticum*, *T. pilulare*, and *T. lappaceum* are rich in phytoestrogenic (plant-based estrogen) compounds (Francis et al. 1967; Vetter 1995).

A plethora of clinical and pre-clinical work demonstrates a multiple of actions associated with red clover and its isoflavones, actions that reflect estrogenic (weak), anti-estrogenic, and estrogen independent mechanisms.

IDENTIFICATION

Botanical Identification

Trifolium is a large genus with many annual and perennial species and subspecies. At least 245 species are recognized worldwide (The Plant List 2013), with approximately 97 listed as either native or naturalized in the United States (USDA NRCS 2016). All *Trifolium* species typically have palmately trifoliate leaves; flowers are gathered in axillary or terminal spikes, or racemes (typically referred to as "heads"); and the corollas are pink, purple, red, white, or yellow. Several species are cultivated for fodder and as green manure in crop rotation, including *T. ambiguum*, *T. fragiferum*, *T. hirtum*, *T. hybridum*, *T. incarnatum*, *T. medium*, *T. pannonicum*, *T. pratense*, *T. repens*, *T. resupinatum*, *T. subterraneum*, and *T. vesiculosum*, among others (Ball 2013; FIS 2009; Kolodziejczyk-Czepas 2012).

Trifolium pratense L. Herbaceous perennial, 3–10 dm, cespitose. **Stems:** Several from base; ascending, branched, hollow, cylindrical; hairy or glabrous. **Leaves:** Palmately trifoliate, leaflets 1.5–6 cm long, elliptic to obovate; approximately 1.2–2.5 times as long as wide, glabrous to pubescent above, pubescent below; alternate; stipulate, stipules oblong, abruptly narrowed to a short awn, usually persistent; lower leaves long-petiolate; upper leave short-petiolate to sessile above. A central blotch or light-colored chevron-like marking is frequently present on the adaxial (upper) surface. **Inflorescence:** Spike (head-like); terminal; sessile or, rarely, short-pedunculate (peduncles shorter than upper leaf petioles); flowers numbering 25–80; spherical, 2–3 cm wide; subtended by two reduced leaves, their stipules forming a false involucre.

Flowers: Sessile; bisexual; papilionaceous; calyx glabrous to strongly pubescent, of five sepals, connate, 10-veined, tube 3–4 mm, lobes subequal with one slightly longer than the others, 3–8 mm; corolla of five petals, zygomorphic, 11–20 mm, pink to reddish-purple, rarely cream or white; ovary superior, monocarpous; stamens 10, diadelphis. **Fruit:** Legume (pod), obconic to obovoid 2–3 mm long; one or two-seeded. **Chromosome number:** 2n = 14.

Varieties and Species: Several varieties of *Trifolium pratense* are recognized including: var. *americanum*, var. *pratense*, var. *sativum*, and var. *villosum* (GRIN 2013). The short-lived var. *sativum* has particularly long stems and large heads and is among the most economically important cultivars (Bailey et al. 1976; Coombe 1968). Cultivars are typically distinguished based on flowering time, yield, and resistance to diseases (Wheaton 1993). Different cultivars are preferred in different geographical regions.

Two species that closely resemble *T. pratense* but are seldom encountered are: *T. medium* (rare introduction) and *T. macraei* (California). Other more common and widespread species of clover, such as white clover (*T. repens*) and alsike clover *T. hybridum* are easily distinguished by the difference of flower color (white or pale pink) and pedicellate flowers.

Distribution: Red clover is native to Europe and was introduced to much of the US and Canada. It is widely cultivated and escaped in fields, roadsides, and disturbed areas. Usually flowers from May to September (up until late November in the Pacific Northwest) (Bailey et al. 1976; Coombe 1968; Gleason and Cronquist 1991; Great Plains Flora Association 1986 [GPFH]; Hitchcock and Cronquist 1973; Vincent and Isely 2012).

Macroscopic Identification

Historically medicinal preparations prepared from red clover consisted of either the inflorescence, flowering tops with subtending leaves, entire aerial parts, or a mixture of entire and broken fragments of the flower heads or aerial parts of *Trifolium pratense*. Today, both blossoms and the entire aboveground parts (leaves, stem, flowers), sometimes without blossoms, are used in commercial preparations (see Figures 4a–o).

A. Leaves

Trifoliate, stipulate, stipules fused to petiole. Stipules broad, membranous whitish with green nerves. Petioles of lowest leaves long, uppermost leaves with very short petioles; leaflets petiolulate, obovate to obovate-oblong or broadly elliptical, 1.5–5 cm in length, 0.7–1.5 cm in width; apex obtuse or retuse, base broadly cuneate; margin ciliate, obtusely serrulate; upper surface glabrous to pubescent, often with a pale chevron-shaped marking; lower surface pubescent.

B. Flowers

When whole, inflorescence ovoid or globular, 1–3 cm in diameter, sessile or rarely short pedunculate, shriveled, purplish to reddish-purple or pink when fresh or properly dried, rarely white, often brownish when improperly dried or harvested late (Figure 6h), composed of numerous papilionaceous (butterfly-shaped) (Figure 3e–h) flowers attached to a central axis (Figure 4l), and often subtended by a pair of modified stipules (Figure 3f), giving the appearance of an involucre; stipules broad, whitish with green nerves. Whole or fragmented simple or trifoliate leaves sometimes attached at stipules. Individual florets (Figure 3m), 1.2–1.8 cm in length; calyx tubular, up to 5 mm in length, pubescent, tube greenish-white, upper edge and teeth green, five-toothed, teeth long, thin, and blunt-pointed, somewhat wiry in appearance, four teeth equal in length, the fifth tooth

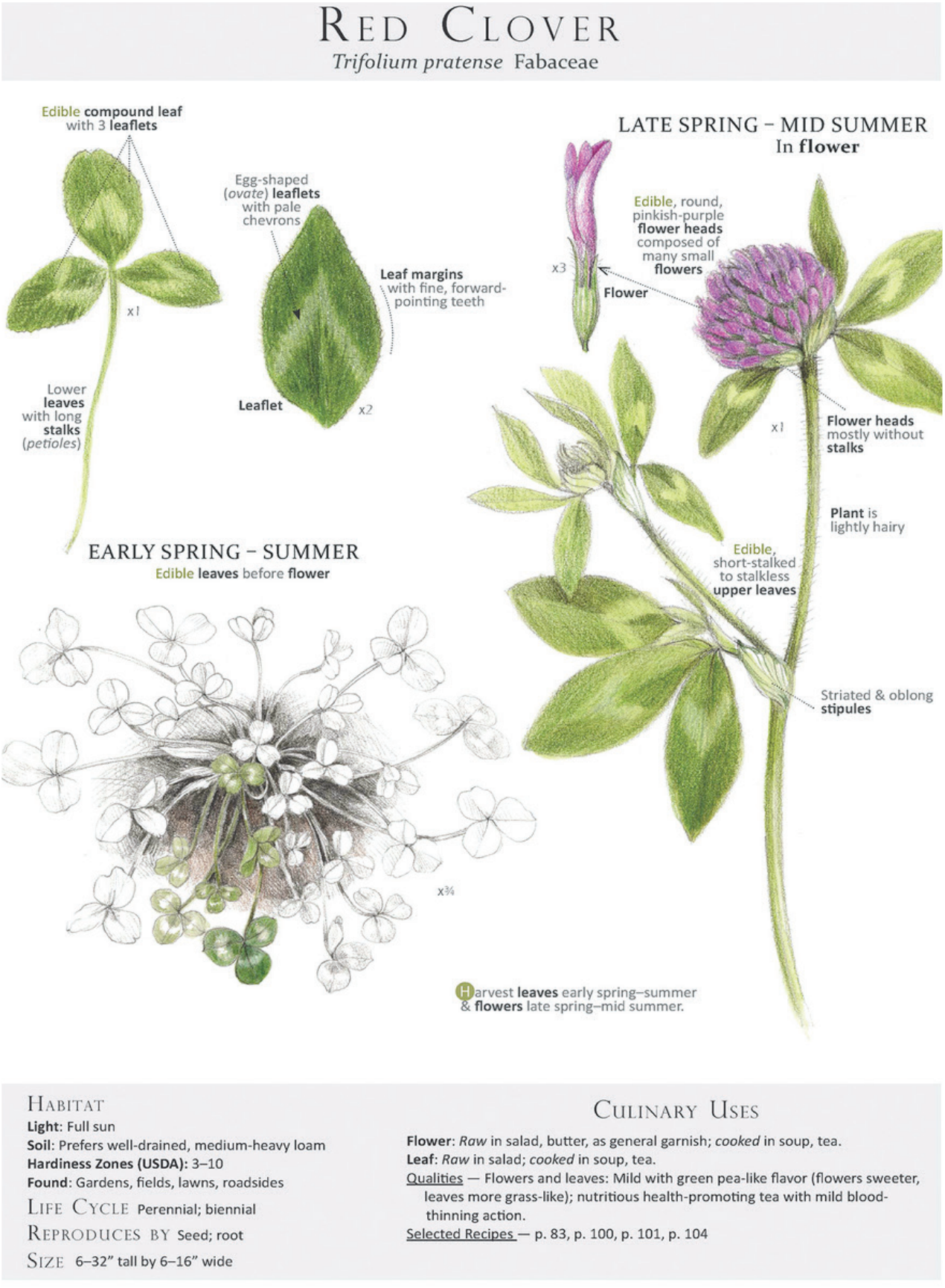

Figure 2 Botanical identification of *Trifolium pratense*

 Source: Foraging and Feasting: A Field Guide and Wild Food Cookbook, Botanical Arts Press, Accord, NY

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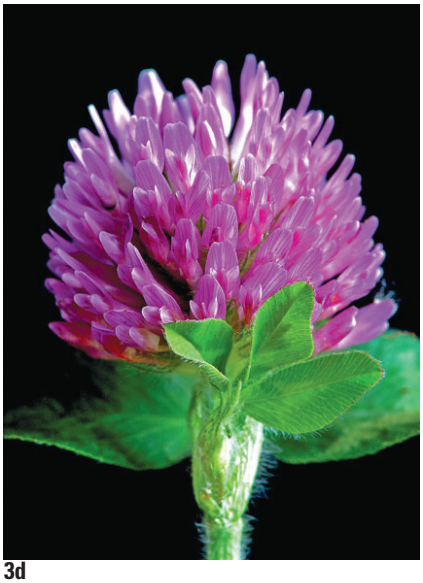

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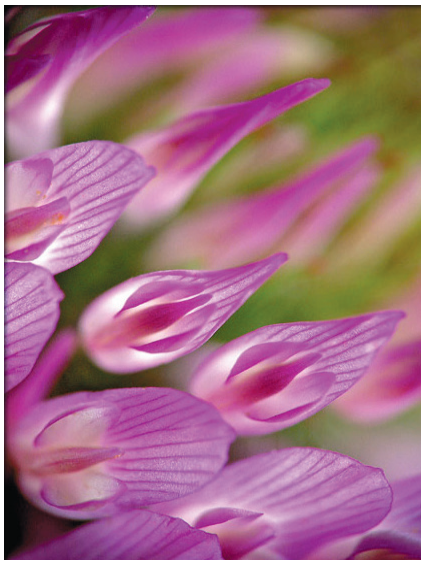

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Photographs 3a–h & m courtesy of Brian Johnston, Toronto, Canada.

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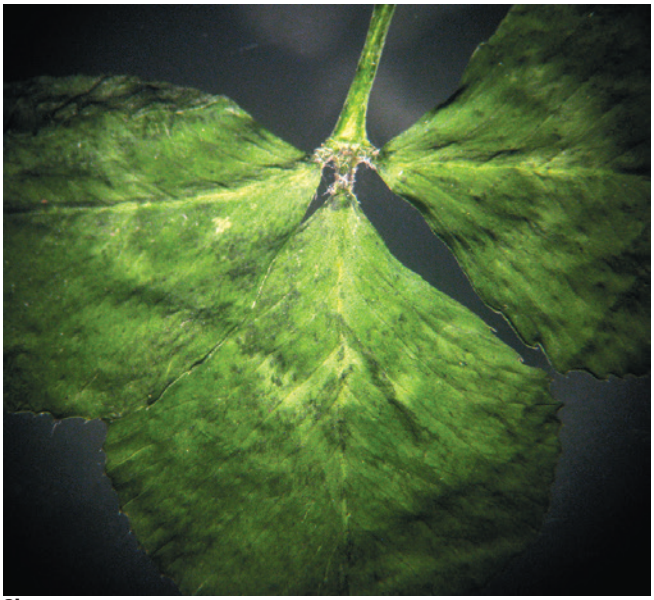

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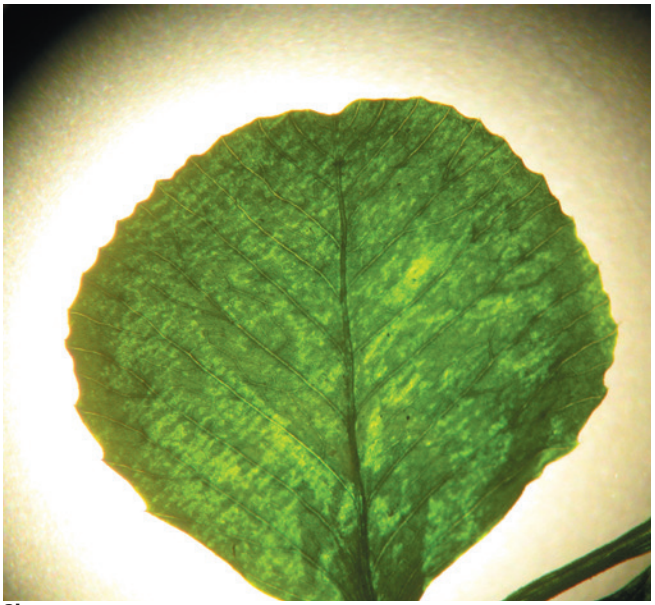

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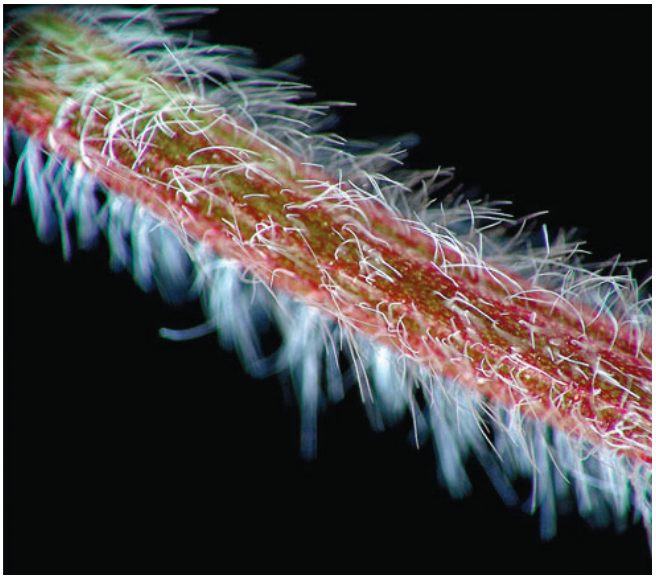
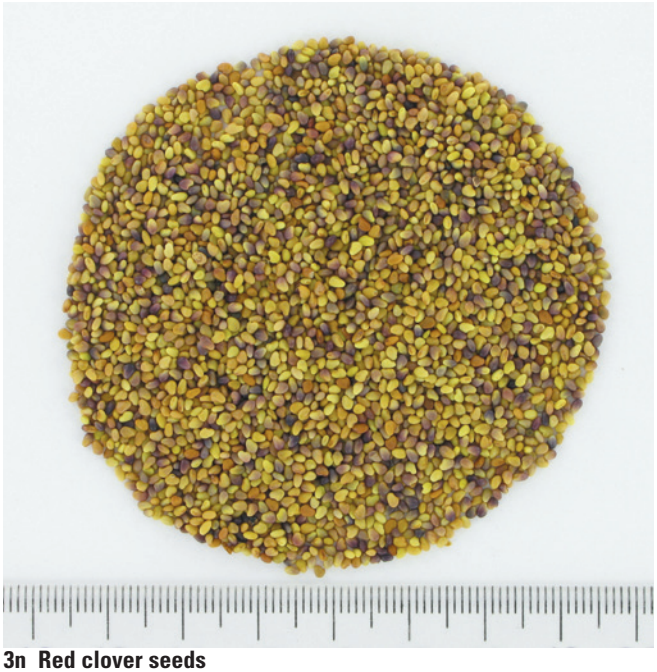


3m

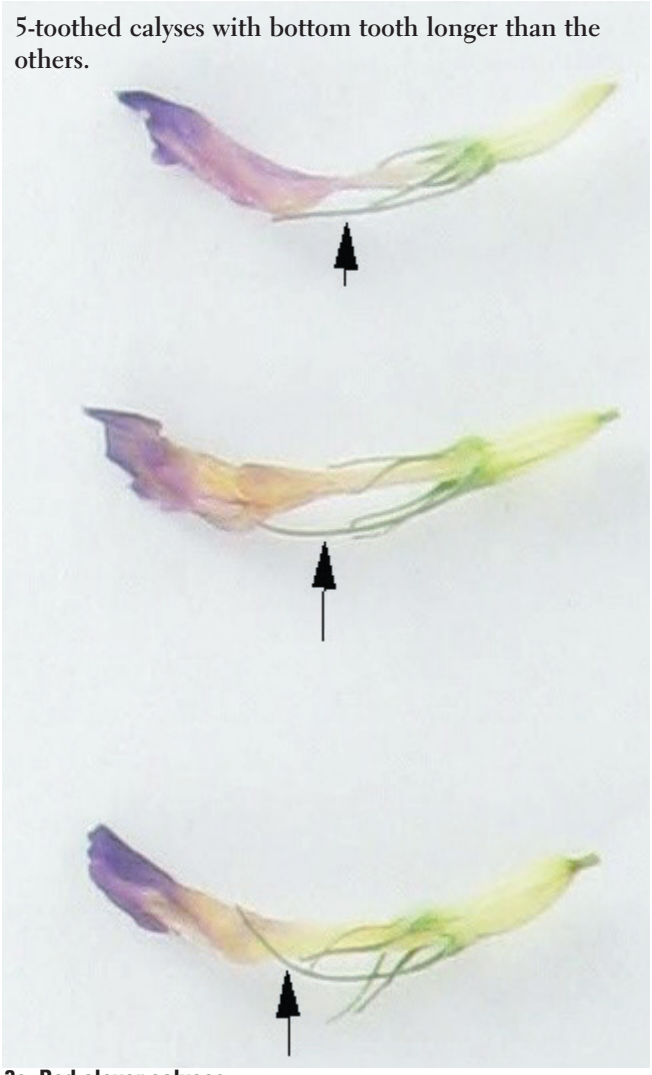
Figures 3a–o Botanical characteristics of *Trifolium pratense*

3a. Red clover blossom emerging.
3b. Red clover blossom emerging.
3c. Red clover blossom.
3d. Fully mature red clover inflorescence.
3e. Red clover inflorescence showing two-winged petals (pappilionaceous).
3f. Inflorescence subtended by highly veinated reduced leaves, their stipules forming a false involucre.
3g. Red clover flowers.
3h. Banner, wing, and keel of red clover flowers.
3i. Red clover leaf showing characteristic chevron.
3j. Red clover leaf showing ciliated margins.
3k. Red clover leaf showing hirsute joint of three leaflets.
3l. Red clover leaflet backlit showing pinnate veination and serrated margin.
3m. Red clover stem showing hirsute covering.

3i & j 7Song, Northeast School of Botanical Medicine, Ithaca, NY; k, l, n-o Lynette Casper, Planetary Herbals, Scotts Valley, CA.



3n Red clover seeds



3o Red clover calyces

much longer than the others (Figure 3o), 10-nerved; corolla five-petaled, petals united into bilaterally symmetrical campanulate tube, somewhat recurved, one standard petal larger and wider than the two free wing petals, two shorter keel petals, base of corolla tube clear-whitish; stamens 10, with nine grouped together and one separate; style slender.

C. Stems
Hollow, cylindrical, may be hairy or glabrous.

Organoleptic Characterization

Texture:	Light.
Fracture:	N/A.
Aroma:	Slight floral, grassy.
Taste:	Slight, at first sweetish then slightly bitter.
Powder:	Slightly aromatic, slightly bitter. Varies widely in color depending on ratio of inflorescences to leaves and stems. Material consisting mainly of inflores cences will appear more pinkish-grey or pinkish-green, with fragments of calyces and florets readily observable under mag nification (10x) (see Figure 4k). In con trast, material consisting mainly of leaves and stems will be greenish, with few to no fragments of florets or calyces.

Microscopic Identification

A. Leaf

Surface view: Upper and lower epidermis similar, except cells on upper surface are rounded to polygonal, while those on the lower surface have wavy anticlinal walls; anomocytic stomata approx. 20 µm long occur on both surfaces; covering trichomes small, three-celled, with a large spherical basal cell, a very small thick-walled second cell, and a long, up to 900 µm, extremely thick-walled acute terminal cell that frequently has a highly narrowed lumen; glandular trichomes occur primarily along veins on the lower surface; they are uniseriate with a short stalk and elongated multicellular head up to 150 µm long; vascular bundles include fibers and are accompanied by a sheath of very small calcium oxalate prism crystals, each ~10 µm long.

Transverse section: Bifacial; palisade cells in 1–3 irregular rows; spongy mesophyll compact, containing calcium oxalate prisms as crystal sheaths along the fibers at the veins.

B. Flower

Calyx: Tube densely covered with appressed, uniseriate, three-celled covering trichomes; very long covering trichomes occur on the apices of the considerably elongated calyx teeth; trichome cell walls heavily thickened with slightly warty cuticle; glandular trichomes frequent on the tubular region of the calyx; calcium oxalate prism crystals, ~8–10 µm long, occur as a sheath along the veins and in the

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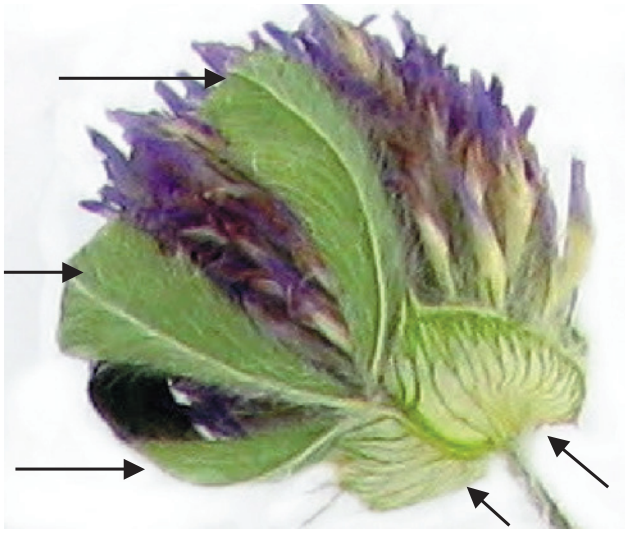

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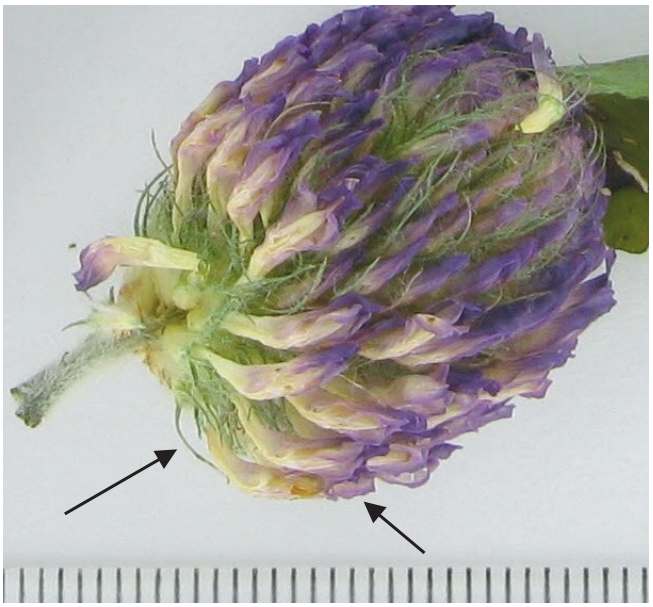

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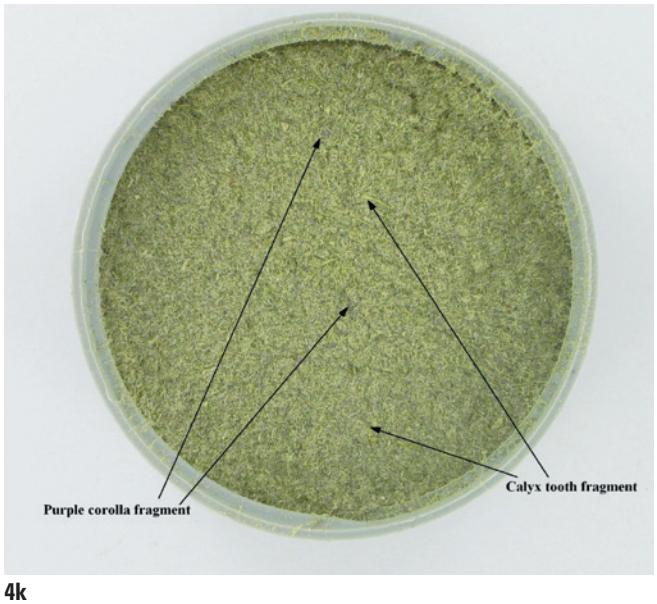

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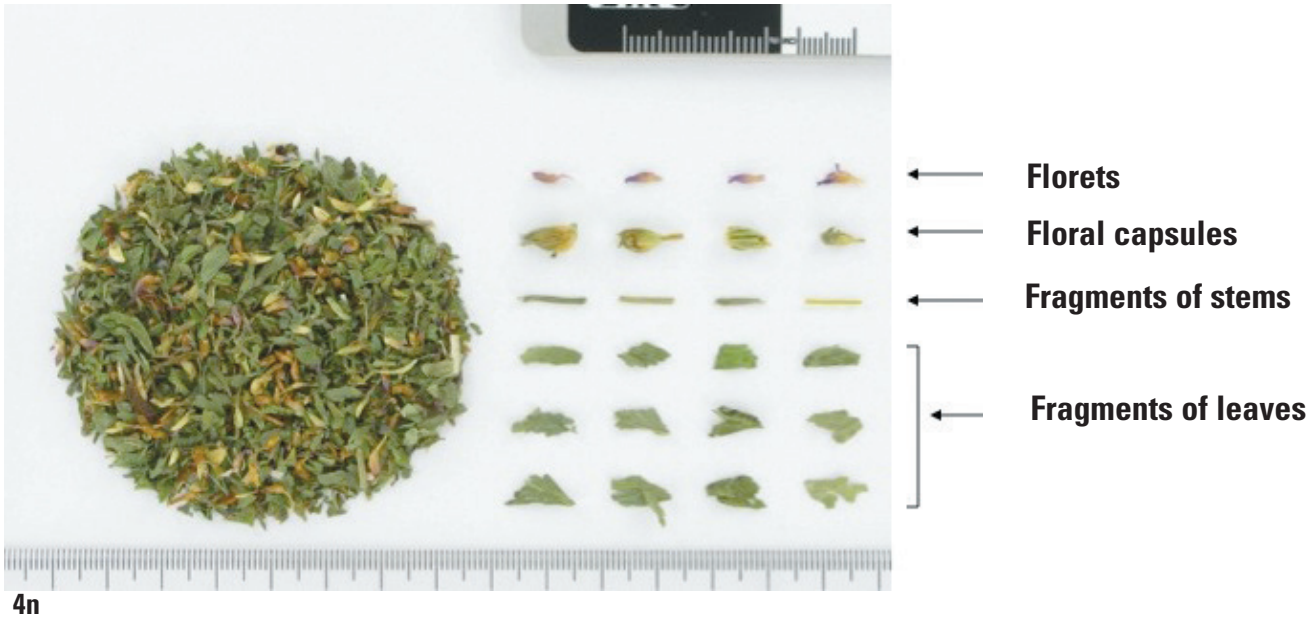


4n

Figures 4a–n Macroscopic characteristics of red clover

4a. *Trifolium pratense* fresh inflorescences with subtending leaves.
4b. Macroscopic characters of *Trifolium pratense* inflorescences.
4c. *Trifolium pratense* aerial parts with inflorescences.
4d. Close-up of *Trifolium pratense* cut and sifted leaf fragments and florets.
4e. Dried *Trifolium pratense* leaflets.
4f. Macroscopic characters of *Trifolium pratense* inflorescences.
4g. Dried *Trifolium pratense* leaves.
4h. *Trifolium pratense* inflorescence showing a subtending trifoliate leaf (left arrows) and stipules (right arrows
4i. *Trifolium pratense* leaf showing typical chevrons that fade with drying.
4j. *Trifolium pratense* inflorescence showing long green calyx teeth (left arrow) and purple florets (right arrow).
4k. Powdered aerial parts of *Trifolium pratense.*
4l. Dissected *Trifolium pratense* inflorescence showing florets attached at central axis.
4m. *Trifolium pratense* inflorescences with fragments of blossoms, stems, and leaves.
4n. *Trifolium pratense* aerial parts (note more leaf than blossoms).

Photographs: 4a courtesy of Linnea SA Riazzino, Switzerland. Lynette Casper, Planetary Herbals, Scotts Valley, CA.

intercostal regions.
Corolla: Epidermal cells papillose with wavy anticlinal walls and a striated cuticle; calcium oxalate prism crystals may cover large areas; trichomes absent.

Pollen: Elliptical, triangular, or sub-spherical in polar view, rectangular-obtuse to rhombic-obtuse in equatorial view, ~25–40 µm in diameter, tricolpate, with a smooth to finely warty exine.

C. Stem

Transverse section: Overall outline shows ridges along the surface; interior to the epidermis is a small ring of collenchyma; inside each ridge lies a large vascular bundle with a huge fiber cap outside the phloem; between vascular bundles is a ring of thickened parenchyma; pith of parenchyma cells.

Powder

Fragments of the calyx with covering trichomes and calcium oxalate prisms; pollen grains; leaf fragments with bases of covering trichomes and veins with calcium oxalate prism sheaths; fragments of the hairless pink corolla; fiber bundles from the stem with calcium oxalate prism sheath (see Figure 5).

COMMERCIAL SOURCES AND HANDLING

Sourcing

Red clover is not native to North America but is a naturalized legume species introduced by colonial migrants. It is widely cultivated and it is also abundant in the wild. **Wild Collection Areas:** Republic of Albania, Republic of Croatia, Bosnia and Herzegovina, Republic of Bulgaria. **Primary Areas of Cultivation (mainly for *Trifolii herba*)**: Federal Republic of Germany, Hellenic Republic (Greece), Republic of Bulgaria; Republic of Croatia, Republic of Serbia, Romania, United States of America (Oregon, Washington), Canada (British Columbia, Québec). Selectively bred varieties developed for optimization of isoflavone content for the manufacture of novel semi-purified extracts are grown in several countries including Australia, New Zealand, French Republic, and Republic of Italy.

In the US several small organic farms cultivate red clover for the herbal market. For dietary supplement purposes, cultivars rich in isoflavones are desired, whereas in agriculture, cultivars are selected based on resistance to diseases (fungus and mold), climate adaptability, stand longevity, and robust seeding.

Red clover is well adapted to a variety of growing conditions, including humid and dry climatic zones, and it is most productive under moderately cool summer temperatures when adequate soil moisture is available throughout the growing season. In North America, the majority of red clover is grown in northern regions of United States (Northeast to the Midwest) and in the central and eastern regions of Canada, and most red clover herbage comes from cultivars developed for livestock use. The indigenous people of North America traditionally used limited quantities of herbage harvested from red clover for medicine. Over the last 20 years, red clover herbage and extracts have increasingly been used as a natural alternative to hormone replacement therapy. Also, since this legume species produces significant concentrations of bioactive isoflavone compounds, it has been commercialized for other nutraceutical purposes. Best practices for growing red clover for nutraceutical purposes are similar to those used to produce animal feed. Detailed information is available in Taylor and Quesenberry (1996) and numerous online sources. To maximize yield, cultivar selection and pest/disease management strategies must be site-specific. For recommendations, local departments of agriculture should be contacted to consult with an extension agronomist specializing in forage crops.

Determinations regarding optimal harvest times should take into consideration both constituent profile and red clover's phytoestrogen activity. A number of studies compared the phytoestrogen content and estrogenic activity of various parts of red clover. Flux et al. (1963) reported the leaf or leaf and petiole had the highest activity, small and large stem fractions were both relatively less active, and blossom and seed head were inactive. Vetter (1995) similarly reported low levels of isoflavones in red clover stems but reported they were rich in daidzein, and sometimes, in biochanin A. One study (Vetter 1995), reported higher isoflavone content in red clover flower heads (1.209 mg/g dw) compared with leaves (1.067 mg/g dw) (Vetter 1995), and another study (Dedio and Clark 1968) reported the highest isoflavone content in leaves prior to flowering (~1.1%), with a rapid decline after flowering and low levels (0.20%) in seeds. The most recent investigations of Booth et al. (2006b) found that total isoflavone content of extracts of red clover aboveground parts were generally much higher than that of extracts of flower heads alone. Additionally, with a few exceptions, this was consistent with the content of the four isoflavones tested, namely, formononetin, biochanin A, daidzein, and genistein, which, in most samples, were higher in total aboveground parts than flower heads alone.

Harvest and Collection Practices

The four primary isoflavones in red clover are in descending concentration: formononetin, biochanin A, daidzein, and genistein (Booth et al. 2006a, 2006b). Based on available data daidzein and genistein exhibit the most pronounced estrogen-modulating activity in cell lines (MCF-7 cells) suggesting these possess the strongest estrogenic-modulating activity. In support of this, red clover samples that had the highest concentration of formononetin and biochanin A exhibited the lowest level of estrogen-modulation (in Ishikawa endometrial cells) (Booth et al. 2006a, 2006b). Additionally, formononetin and biochanin A are metabolically (demethylated) converted to daidzein and genistein,

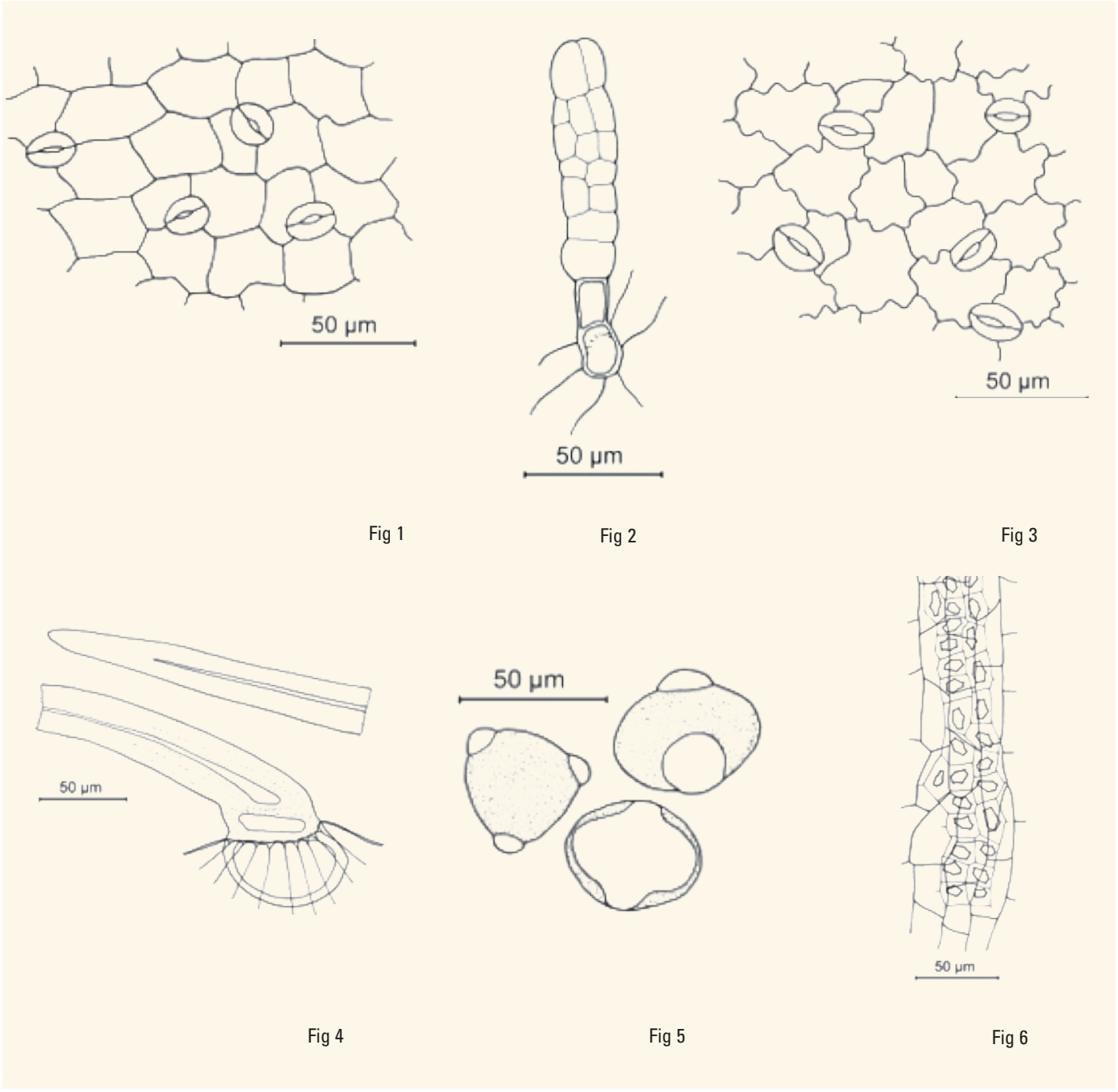
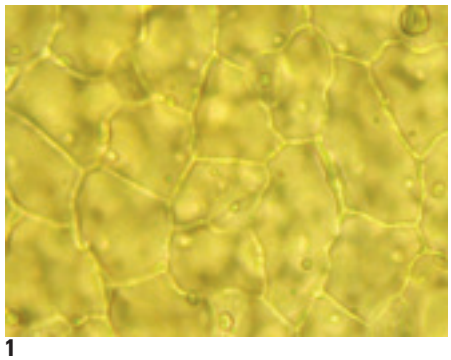


Figure 5a Microscopic characteristics of red clover (illustrations)

1. Leaf upper epidermis showing anomocytic stomata (sv).
2. Multicellular glandular trichome from the calyx (sv).
3. Leaf lower epidermis showing anomocytic stomata (sv).
4. Three-celled covering trichome from the leaf upper epidermis (sv).
5. Tricolpate pollen grains.
6. Calcium oxalate prism sheath along a vein on the leaf lower epidermis (sv).

respectively, in vivo (Heinonen et al. 2004), while genistein can be converted to equol, a substance known to affect many estrogenic responses in cell lines, including growth stimulation, estrogen receptor (ER) binding, and downregulation of ER mRNA (Sathyamoorthy and Wang 1997; Welshons et al. 1987). Prunetin, another compound in red clover is similarly metabolically converted into genistein (Hu et al. 2003). Caution must be exercised when interpreting results of investigations with cell lines as metabolization of compounds being tested can differ between in vitro estrogenic assays (Booth et al. 2006b).

Data regarding seasonal fluctuations of isoflavone content in red clover differ. This is partly due to fluctuations in environmental conditions and plant strain as well due to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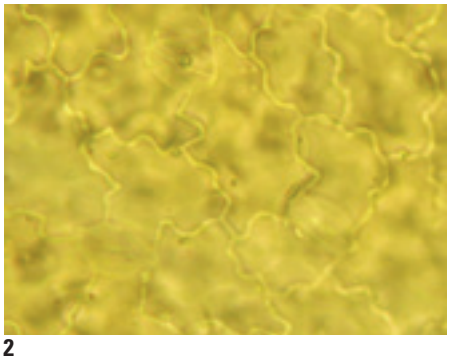
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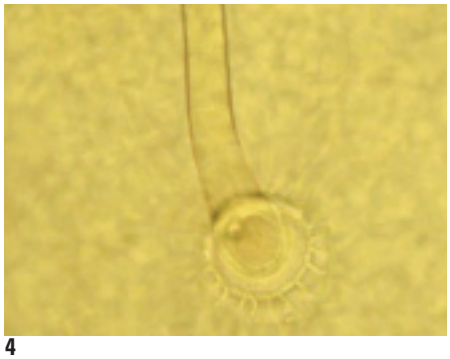
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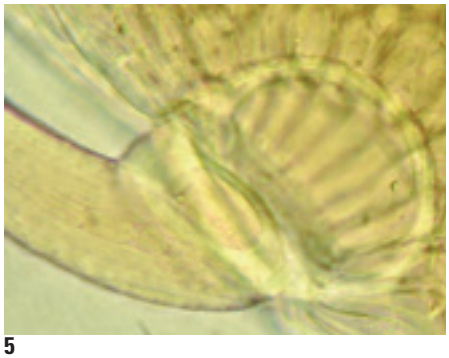
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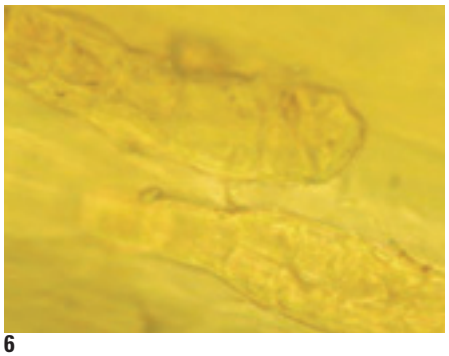
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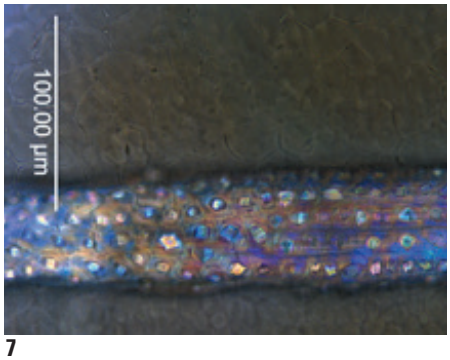

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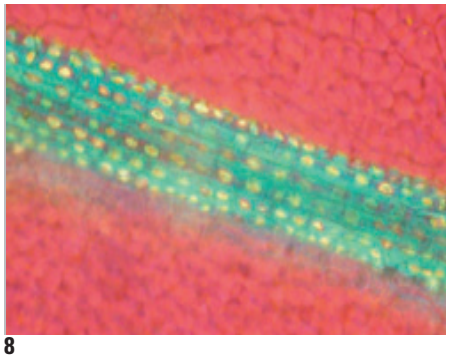
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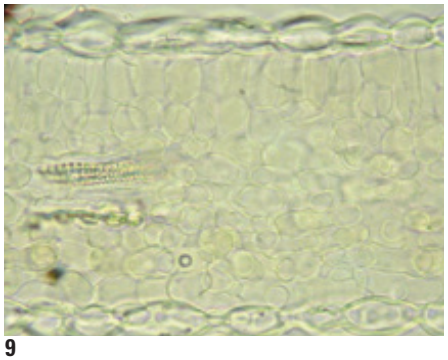
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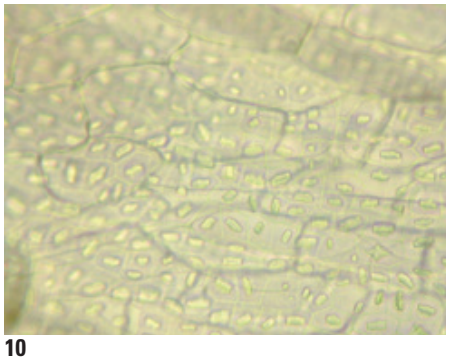
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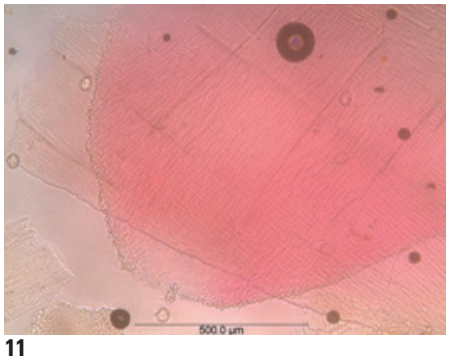

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Figure 5b Microscopic characteristics of red clover (images)

1. Leaf upper epidermis showing rounded to polygonal cells (sv).
2. Leaf lower epidermis showing wavy anticlinal walls (sv).
3. Tapering trichome with warty exine
4. Base of a three-celled covering trichome on the leaf upper epidermis (sv).
5. Base of a three-celled covering trichome on the leaf margin (lv).
6. Multicellular club-shaped glandular trichomes along a vein on the leaf lower epidermis (sv).
7. Calcium oxalate prism sheath along a vein on the leaf lower epidermis (sv).
8. Calcium oxalate prism sheath along a vein on the leaf lower epidermis (polarized light, compensator first order) (sv).
9. Leaf transverse section showing the bifacial structure.
10. Calcium oxalate prisms in an intercostal region of the calyx (sv).
11. Fragment of floret showing red color reaction to acidified chloral hydrate glycerin solution.
12. Tricolpate pollen grain with a finely warty exine.

differences in the analytical techniques used. Early methods used to quantitate isoflavones in red clover were based on TLC or paper chromatography, using densitometric or fluorometric detection (Dedio and Clark 1968; Francis et al. 1967; Vetter and Nagy 1991) in contrast to analyses with HPLC (e.g., Booth et al. 2006a, 2006b; Kallela et al. 1987; Krenn et al. 2002; Wu et al. 2003) and UPLC (Lemeziene et al. 2015).

The results of Booth et al. (2006b) using HPLC showed that formononetin, biochanin A, daidzein, and genistein in most all samples, were, significantly higher in extracts of aboveground parts compared with extracts of only flower heads, except that the content of biochanin A in a single sample and the content of genistein in a single sample were anomalously higher in flower heads than aboveground parts. Further, according to the same analyses, daidzein and genistein appeared to peak around July, while formononetin and biochanin A levels peaked in early September. Formononetin and biochanin A represent approximately 90% of the total isoflavones in red clover. Based on total isoflavone concentration, the researchers concluded that optimal harvest for red clover aboveground parts is early September, at the end of the blooming season. They further suggest that harvest of flowering heads in July and aboveground parts in September might be considered. These findings are consistent with those of Tsao et al. (2006). Varieties of red clover grown in different geographic locations may present a different seasonal variation of isoflavone content.

Another detailed analysis of red clover genotypes was conducted to determine isoflavone concentration at different stages of growth specifically to identify optimal harvest times and preferred genotypes for potential use in the dietary supplement market. The highest concentration of formononetin (51%) and biochanin A (40%) occurred when flowering. Concentrations of formononetin, biochanin A, daidzein and genistein at flowering stage ranged between 2.61 and 4.40, 1.79 to 3.32, 0.06 to 0.14, and 0.36 to 0.59 mg/g (dry weight), respectively. The average concentration of total isoflavones based on these primary four at flowering stage during two years of investigations was 6.66 mg g^{-1} and ranged from 5.4 and 8.09 mg/g (dry weight). The highest concentration of total isoflavones at flowering stage occurred in leaves. The average total concentration of the four isoflavones were: 12.29 mg/g in leaves, 2.93 mg/g in stems, and 1.42 mg/g in flowers (dry weight). Genotypes yielding the highest concentrations of isoflavones included Radviliai (8.09 mg/g) and Vyliai (7.29 mg/g). Ploidy level had no significant correlation with isoflavone concentration (Lemeziene et al. 2015; Sivesind and Seguin 2005).

Collection Practices: When harvesting red clover, the plant should be handled carefully to prevent bruising of the tissues, which results in a browning of the blossoms when dried. When harvesting blossoms, the inflorescences and subtending leaves are typically picked (red clover flowering tops), carefully clipping the blossom from the stalk without touching the blossom. Similarly, to prevent bruising, the blossoms should not be compressed into containers too tightly. Some wildcrafters recommend harvest of flowering tops in the morning as the moisture is thought to form a protective barrier around the flowering head (Drum 2013, personal communication to AHP, unreferenced).

For red clover flowers, manual harvesting of the inflorescence (whether farmed or wild harvested) are separated from the other aerial parts. For red clover herb, the entire plant is machine harvested when cultivated and manually harvested in the wild.

Cultivation

Genetics, growing conditions, agronomic practices, postharvest storage, and processing conditions can significantly alter the composition and concentration of polyphenols in fresh and processed red clover herbage. Red clover cultivars are classified according to regional adaptation: 1) Biennial versus short-lived perennial; and 2) Late-flowering versus early flowering (known in Canada as single-cut versus double-cut). Cultivars specifically bred for resistance against plant pathogens are under development. In North America, the majority of currently grown cultivars are multiple-cut diploid and were released as a result of basic research conducted in public institutions and universities. More recently, however, tetraploid cultivars developed for the European market have become available in North America. In light of many studies indicating that isoflavones have a positive impact on human and animal health, recent research has evaluated the diversity of these compounds among currently available red clover cultivars (Tsao et al. 2006). Individual isoflavone concentrations are affected by genotype, harvest date, and year. Seasonal herbage yield varied depending on location and production year, ranging from 8 to 14 metric tons per hectare. First growth during the growing season accounted for about 50% of total seasonal biomass when harvested at full bloom. Even though significant variability has been reported among modern red clover cultivars under greenhouse and field conditions, all cultivars contained high to very high total and individual isoflavone concentrations (Tsao et al. 2006).

In Canada under field conditions, herbage from first growth has been found to have substantially higher total isoflavone concentrations (15 mg/g) than herbage obtained from re-growth (9 mg/g) (Papadopoulos, unpublished data). To maximize bioactive isoflavone compound content, red clover should be harvested between early flowering and full bloom. In general, the highest levels of various isoflavone concentrations are found in the leaves, followed by the stem, petiole, and flower, respectively (Tsao et al. 2006).

Red clover (*Trifolium pratense* L. cv. Kenland) was found to be an Iron (Fe)-efficient plant while it was sensitive to deficiencies of copper (Cu), zinc (Zn), and manganese (Mn) or toxicities of Cu and Mn (Zheng et al. 2003). Application of phosphorus (P) increases plant growth (see Sainz and Arines 1988).

A plethora of cultivation information is available on a variety of online agricultural resources.

Plant Diseases

There are a host of diseases to which red clover is susceptible. Although red clover is a perennial, production levels decline dramatically two years after sowing due to a variety of diseases including root rot and infestations of the root borer *Hylastinus obscurus* (Palma et al. 2012). Detailed information on diseases in clovers as well as integrated pest management (IPM) programs for disease control are provided in a variety of online agricultural sources.

Conservation Status

The International Union for Conservation of Nature (IUCN) 'European Red List of Medicinal Plants' assigns *Trifolium pratense* to the conservation category of 'Least Concern' (LC), meaning that the species is not threatened in Europe.

Handling and Processing

Crushing plant material prior to extraction results in higher yields of isoflavone aglycones compared with their glycosides (Francis et al. 1967), due to enzymatic autohydrolytic release of aglycones from the stored glycosides within the plant. This is another likely source of variation when quantitating isoflavones in red clover.

Drying

Data regarding optimum drying conditions are lacking. In the analytical work of Booth et al. (2006b), samples were dried for at least 10 days at ambient temperature in a shaded greenhouse that excluded 60%–70% of sun. As with most legume fodders (e.g., alfalfa [*Medicago sativa*] and clover), care must be taken to carefully dry the material without molding. Negative effects observed in animals eating clover are due to the consumption of moldy clover. Signs of mold, such as a rust coloring on the upper side of the leaf caused by *Rhizoctonia leguminicola* or black coloring on the underside of the leaf caused by *Cymodothea trifolii*, is usually visible (Murphy 2016).

When drying flowering tops, layer no more than three blossoms deep or the weight of the top blossoms will press those on the bottom, compressing and damaging tissue resulting in browning of the blossoms. Sometimes, the flowers are carefully turned for even drying (Drum 2013, personal communication to AHP, unreferenced). Some recommend turning the blossoms frequently during the drying process to prevent discoloration (Moore 2003).

In one study, freeze-drying (-80 °C overnight after nitrogen treatment and vacuumed dried for 48 h) inhibited the conversion of glycosides to aglycones, while drying under vacuum resulted in a maximum conversion of glycosides to their corresponding aglycones. Drying by air (ambient) produced a low level of the aglycones formononetin and biochanin A, while oven drying (100 °C) promoted decarboxylation of the malonyl glucosides to the acetyl glucosides. Exposure to UV-B radiation resulted in an increase in formononetin and biochanin A, caffeic acid (40%), and flavonols (250%). Freeze-drying resulted in a constituent profile that was most consistent with the naturally occurring profile (Swinny and Ryan 2005) (see Figure 6f).

Storage

Protect from air, heat, light, moisture, and insects. There is little literature on optimal storage conditions for red clover. In one study the isoflavone concentration in fresh plant material was 2,050, 1,766, 306, and 127 µg/g (dry weight) for formononetin, biochanin A, genistein, and daidzein, respectively. After four days of drying in the field, no significant change in isoflavone concentration was found, except for daidzein, which increased two-fold. Follow-up experiments with laboratory-scale silos, showed a decrease in isoflavone concentration during the first two weeks, followed by stabilization over the five remaining months of the experiment. The concentrations fell by 26%, 39%, 66%, and 73% for daidzein, genistein, biochanin A, and formononetin, respectively (Daems. 2016).

Qualitative Differentiation

There are varying qualities of red clover ingredients available including aerial parts, flowering tops, and extracts. Much of the commercial red clover on the market consists of inflorescences that are more brown in color than red, indicating suboptimal harvest, handling, drying, or storage conditions. When considering red clover for isoflavone content, current literature consistently reports the highest concentrations in leaf, stems, and inflorescences (Sievesind and Seguin 2005; Tsao et al. 2006).

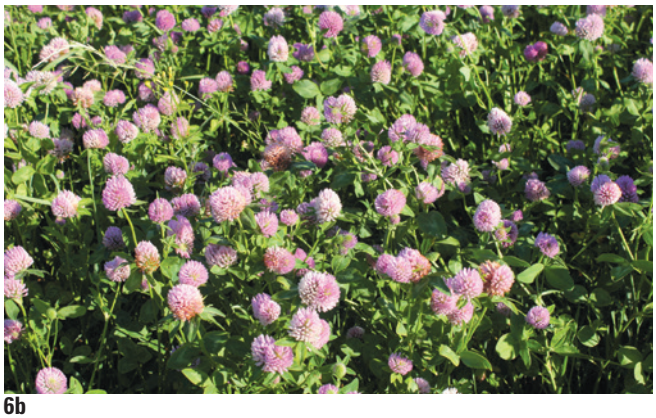
Substitutions and Adulterations

None reported. Most *Trifolium* species are considerably different botanically, and should not be mistaken by experienced herbalists and harvesters. The isoflavone content of a number of these *Trifolium* species has been reported. *Trifolium montanum* (0.359 mg/g dw), *T. incarnatum* (0.859), *T. fragiferum* (0.246), and *T. repens* (white clover; 0.24) all yield low concentrations of isoflavones compared to red clover (3.02 mg/g dw). Other species of *Trifolium* yielded concentrations of isoflavones comparable to and higher than *T. pratense*, namely, *T. alpestre* (3.759) and *T. subterraneum* (3.657) (Vetter 1995). Oleszek et al. (2007) reported on the analysis of 57 species of *Trifolium* and found the isoflavone content of *T. heldreichianum*, *T. scabrum*, and *T. subterraneum* to be 7% to 9% (dw), much higher than his results for *T. pratense* (1%–4%).

Preparations

There are a limited number of red clover products on the market and predominantly include blossoms and whole plant used in tea mixtures, tinctures, and dry extracts. Dry extracts prepared from aerial parts (mostly leaves and stems) are the only preparations subjected to clinical trials, predominantly for the treatment of menopausal symptoms. The material primarily used in clinical trials is cultivated in France and Germany adhering to good agricultural practices and is grown specifically for manufacture of the extract rather than as a secondary product for fodder. For this extract, two different different seed lines are used: one selected for high biochanin A yields; the other for high formononetin yields. For traditional preparations see Red Clover Formulas.

6a



6b

6c

6d

Figure 6 Commercial Sources and Handling

6a Red clover in meadow.
6b Red clover cover crop.
6c Field of red clover leaf and stem prior to harvest.
6d Mechanical harvest of red clover leaf and stem.
Photographs: 6a courtesy of Todd Caldecott, Dogwood School of Botanical Medicine, Toronto, Canada; 6b Henriette's Herbal Homepage: www.henriettes-herb.com; 6c & d courtesy Linnea SA Riazzino, Switzerland

CONSTITUENTS

The primary constituents of interest in red clover (*Trifolium pratense*) for which activity has been demonstrated are isoflavonoids, which can be subdivided into isoflavones and pterocarpans. Additionally, flavonoids, such as quercetin, are also present along with polyphenolic amides and phenolic acids and their derivatives (i.e., clovamide and phaselic acid). The isoflavones have been isolated in their aglycone forms or conjugated to glycosides and glycoside malonates. Isoflavones in red clover mainly consist of glycosides and malonyl-glycosides. Malonyl esters of isoflavone glycosides are not stable during processing, while their corresponding glycosides are stable. The glycosylated compounds are considered biologically inactive, because of their inability to cross cell membranes (Rijke et al. 2001; Simonne et al. 2000; Tsao et al. 2006). Aglycones, in contrast, are considered the bioactive forms of the isoflavones, due to their rapid rate of absorption and transport across human intestinal epithelial cell monolayers (Izumi et al. 2000; Steensma et al. 1999). Other potentially bioactive constituents in red clover include those contained in the volatile oil, e.g., alcohols, ketones, and terpenes. Among the major groups of phenolics present in red clover, isoflavones are considered key bioactive constituents possessing estrogenic properties that may play a role in cancer prevention, moderation of menopausal symptoms, and other health effects (Tham et al. 1998).

Phytochemical profiles and their distribution vary based on origin of the plant (cultivars), cultivation influences (e.g., fertilization), different parts of the plant, and growing stages (Booth et al. 2006b; Tsao et al. 2006). Flowers contain flavones as the major flavonoids, whereas the leaves contain isoflavones as the major flavonoids (Lin et al. 2000), and flowers are rich in procyanidins (Sivakumaran et al. 2004).

According to one report, red clover adapts to increased levels of ozone by increasing total phenolic concentrations, and thereby, the plant's antioxidant protection against ozone-mediated oxidation (Saviranta et al. 2010). Antioxidant activity is generally correlated with total phenolic and flavonoid contents (Esmaeili et al. 2015).

Isoflavones

Isoflavone aglycones

Isoflavones are categorized as phytoestrogens due to their structural similarity to β-estradiol. The structure of isoflavones is characterized by a 3-phenyl-chromen-4-one core and various substituents such as methoxyl groups, hydroxyl groups, and sugars, often present as acetyl- or malonyl esters. According to most reports, the isoflavone content decreases in the various plant parts from leaf to root, to stem, to

Figure 6e Proportion (%) of formononetin, biochanin A, daidzein, and genistein of all red clover genotypes at flowering stage (2013–2014)

Source: Lemeziene et al. 2015.

flower (Tsao et al. 2006) (see Tables 2 & 3). Ten isoflavone aglycones were identified in all cultivars and all parts in this study (Tsao et al. 2006). These included biochanin A, formononetin, daidzein, genistein, glycitein, irilone, orobol, pratensein, pseudobaptigenin, and prunetin. Similarly, Wu et al. (2003) separated and quantified 10 isoflavone aglycones after hydrolysis, and the composition differed only by the fact that calycosin was found instead of orobol. Researchers report varying values depending on the analytical method used and material assayed (see Peng and Ye 2006; Zgórka et al. 2011). Freshly dried material is reported to have 22% higher concentrations of isoflavones (in mg/g of dry weight) than silage and hay and may be desired when isoflavone concentrations are desired (Sivesind and Seguin 2005). Differences in quantitative values reported in the literature reflect intraspecies variation, differences in analytical methodologies, and differences due to harvest and handling conditions or environment. In addition to the primary phytoestrogenic compounds above, Mazur (1998) reported coumestrol concentrations of 105 to 1570 µg/100 mg dry weight.

Minor isoflavone glycosides identified in red clover include texasin-7-*O*-β-D-glucoside-6″-*O*-malonate, and afrormosin-7-*O*-β-D-glucoside-6″-*O*-malonate (Klejdus et al. 2001). Of the individual isoflavones, formononetin and biochanin A and their glycosides are predominant, and are present at high concentrations in the leaves (Krenn et al. 2002; Saviranta et al. 2008; Tsao et al. 2006; Wu et al. 2003).

According to Tsao et al. (2006), when the entire plant is analyzed at the early budding stage, formononetin and biochanin A concentrations are 8.22 and 7.94 mg/g dry weight, respectively, and increase in the late flowering stage along with total isoflavone concentration by more than 30% (Tsao et al. 2006). The leaves are rich in formononetin and biochanin A (5.57–9.05 and 10.94–14.59 mg/g, respectively). However, the highest concentrations of biochanin A were found in young leaves (Saviranta et al. 2008).

There are also diploid and tetraploid forms of red clover. However, on average, both yield similar concentrations of total isoflavones (Tsao et al. 2006).

Daidzein, genistein, formononetin, biochanin A, coumestrol, and naringenin all display estrogenic activity

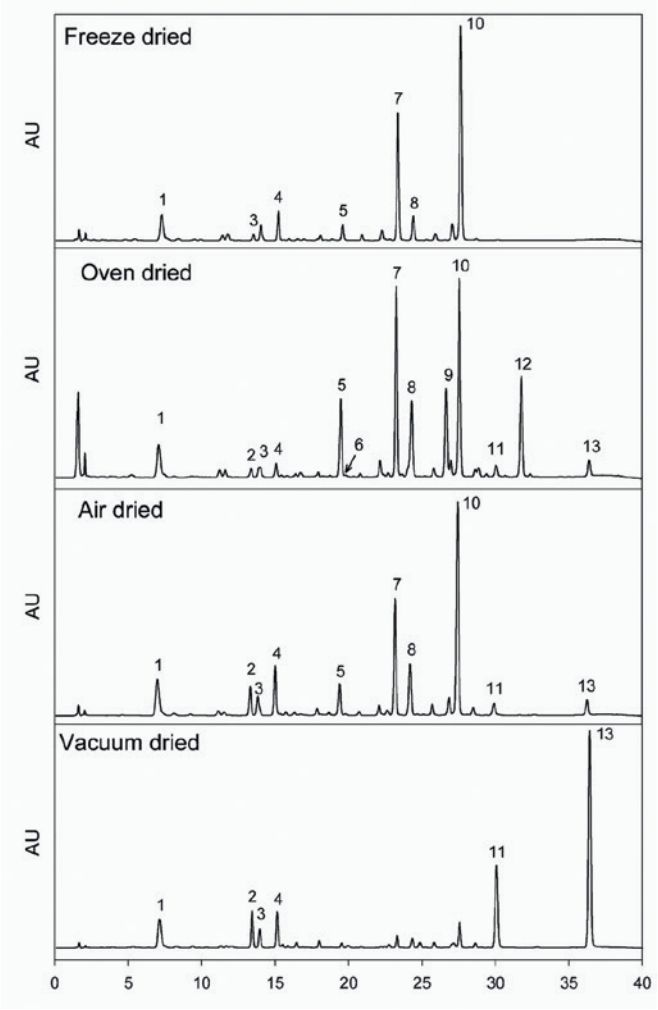


Figure 6f Effect of drying conditions on isoflavone glycoside conjugates.

Peak Identification: 1 = caffeic acid; 2–4 = flavonols; 5 = formononetin glycoside; 6 = daidzein; 7 formononetin acetyl glycoside; 8 = biochanin-A glycoside; 9 = formononetin acetyl glycoside; 10 = biochanin-A malonate glycoside; 11 = formononetin; 12 = biochanin-A acetyl glycoside; 13 = biochanin-A. The freeze-dried sample was harvested later in the season than other samples. **Source:** Swinney and Ryan 2005.

in the alkaline phosphatase (AP) estrogenic assay. All of these, except formononetin, bind to one or both estrogen receptors (ER) (Booth et al. 2006a, 2006b). Coumestrol, first identified in alfalfa (*Medicago sativa*), has a similar binding affinity for the ER-β estrogen receptor as 17β-estradiol, but much less affinity for 17α-estradiol, although the estrogenic potency of coumestrol at both receptors is much less than that of 17β-estradiol (Kuiper et al. 1998).

Isoflavone glycoside and glycoside malonate derivatives

Red clover contains numerous isoflavones mainly in the form of glycosides (e.g., genistin, glycitin, daidzin, ononin, etc.) with or without malonyl or acetyl esters (Rijke et al. 2001; Wu et al. 2003). Although the glycosides and malonylglycosides in plants are generally considered to lack activity, they are of biological interest as they are utilized in plants

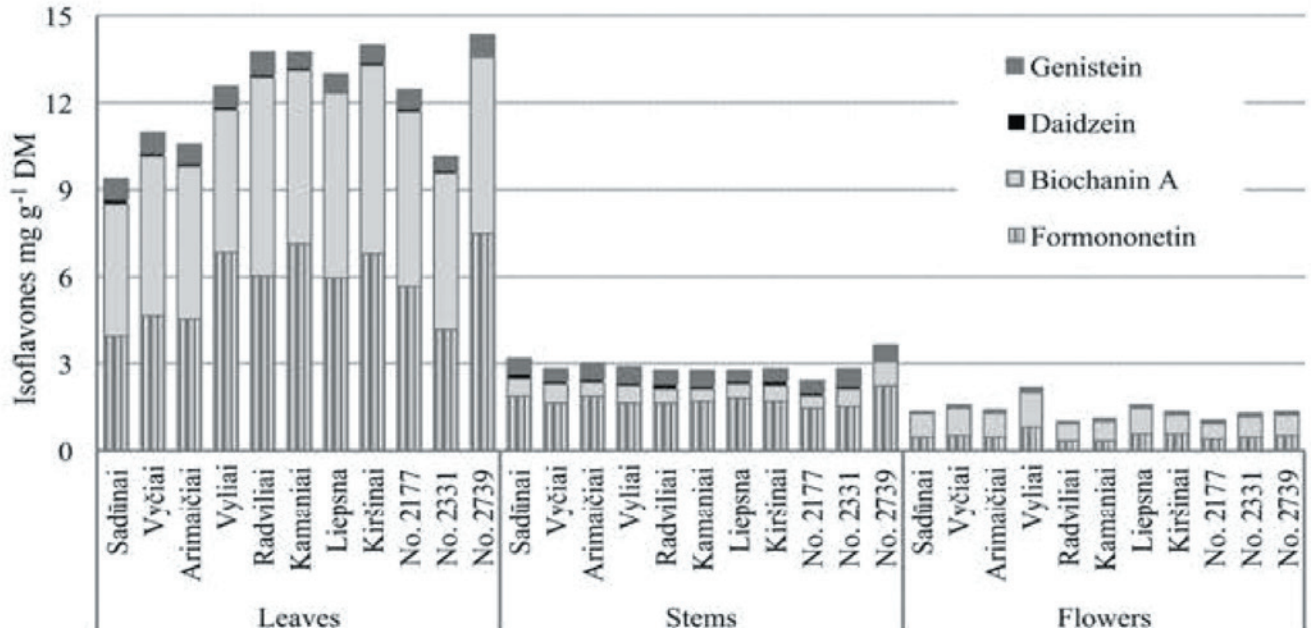


Figure 6g Total isoflavone (formononetin + biochanin A + daidzein + genistein) concentration in the various parts of red clover genotypes (2014) Source: Lemeziene et al. 2015.

to store less-soluble isoflavone aglycones. Upon enzymatic activation, hydrolysis releases the aglycones from their stored conjugated form, allowing them to perform their role as phytoalexins. Of relevance to human use, studies indicate that isoflavone bioavailability is greater when ingested as glycosides (daidzin, genistin), rather than as aglycones (daidzein and genistein) (Setchell et al. 2001).

In red clover leaves and flowers, numerous isoflavone glycosides along with glycoside malonates have been reported (Figure 1) (Klejdus et al. 2001; Lin et al. 2000; Tsao et al. 2006; Wu et al. 2003). However, post-harvest processing and drying methods can affect isoflavone composition, partially transforming isoflavone glycosides into aglycones (Swinny and Ryan 2005).

Pterocarpans

Pterocarpans are derivatives of isoflavonoids found in the

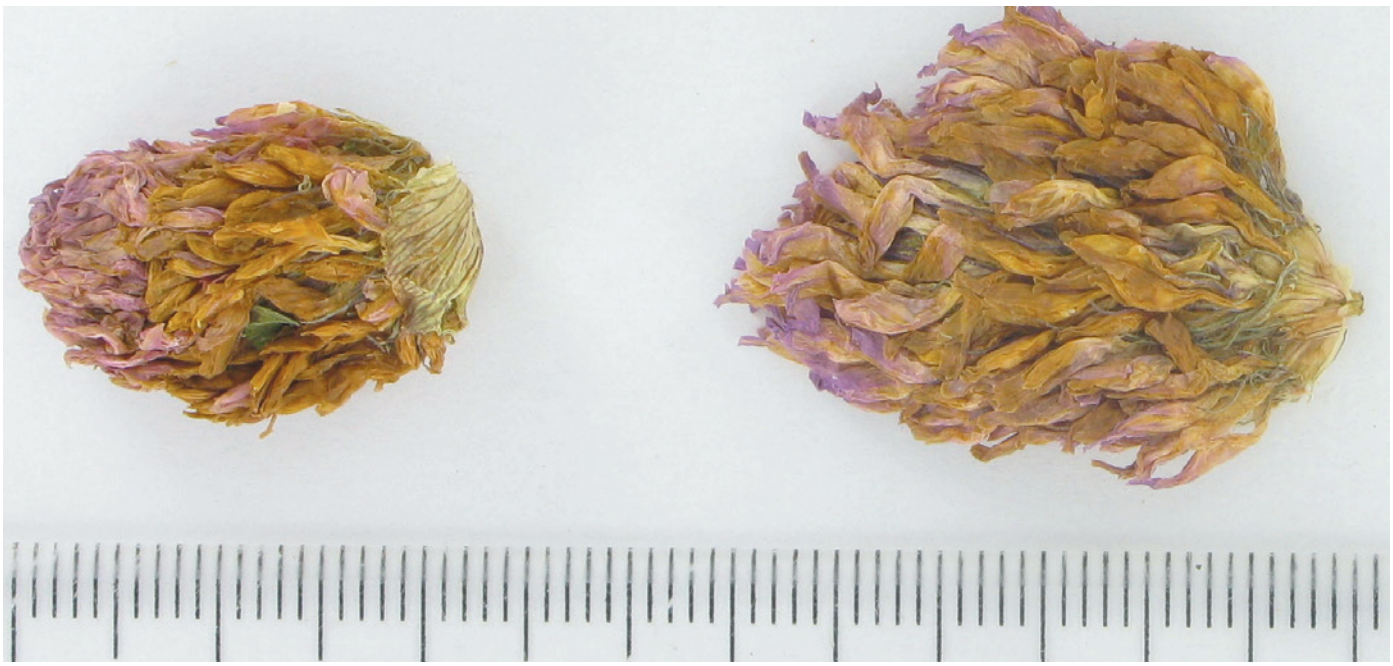


Figure 6h Poor quality red clover inflorescences, brown in color from suboptimal harvesting, drying, or storage

Photograph: Lynette Casper, Planetary Herbals, Scotts Valley, CA.

Table 2 Isoflavone content of *Trifolium pratense* (mg/g dw)*

Leaf	17.2–32.9
Total Isoflavones	0.0–0.94
Daidzein	0.0–0.75
Genistein	5.74–17.9
Biochanin A	6.07–13.3
Formononetin	
Stem	11.2–23.9
Total Isoflavones	0.03–0.46
Daidzein	0.10–0.53
Genistein	1.34–3.95
Biochanin A	6.38–17.51
Formononentin	
Flower	1.68–3.14
Total Isoflavones	0.004–0.005
Daidzein†	0.02–0.12
Genistein	0.61–1.24
Biochanin A	0.47–1.12
Formononentin	
Root†	13.6–28.5
Total Isoflavones	0.12–0.44
Daidzein	1.0–5.8
Genistein	0.67–3.39
Biochanin A	0.19–0.96
Formononentin	

* Calculated as aglycones after hydrolysis HPLC-DAD-MS. **Source:** Tsao et al. 2006. † Analyzed by Wu et al. 2003 (UV detection).

Table 3 Concentrations of minor isoflavones found in the leaves of *Trifolium pratense* (mg/g dw)

Glycitein	1.03–5.96
Pratensein	0.82
Prunetin	0.45
Orobol	0.28
Pseudobaptigenin	0.19
Irilone	0.06

Source: Tsao et al. 2006.

Fabaceae family of plants to which red clover belongs. The pterocarpan glycoside, trifolirhizin, was the first reported antifungal compound found in red clover by Virtanen et al. (1957). Trifolirhizin occurs mainly in the root (Bredenberg and Hietala, 1961) with traces detected in the leaves (Chang et al. 1969). In infected plant tissue, trifolirhizin is converted to its aglycone known as maackiain (Suginome 1962). The occurrence of another pterocarpan, medicarpin, has also been reported in red clover leaves (Booth et al. 2006; Debnam and Smith 1976; Higgins and Smith 1972).

Flavones

Flavonol quercetin and its derivatives, quercetin-3-*O*-(6″-*O*-malonyl)-glucoside and 3-methylquercetin-7-*O*-β-D-glucoside, have been identified in red clover leaves (Swinny and Ryan 2005) and flowers. The following flavone values have also been reported: hyperoside (quercetin 3-*O*-β-D-galactoside) (He et al. 1996; Lin et al. 2000), kaempferol (0.8 mg/g), luteolin (16.6 mg/g), and myricetin (0.5 mg/g) all on a dry weight basis (Tundis et al. 2015).

Phenolic Acids

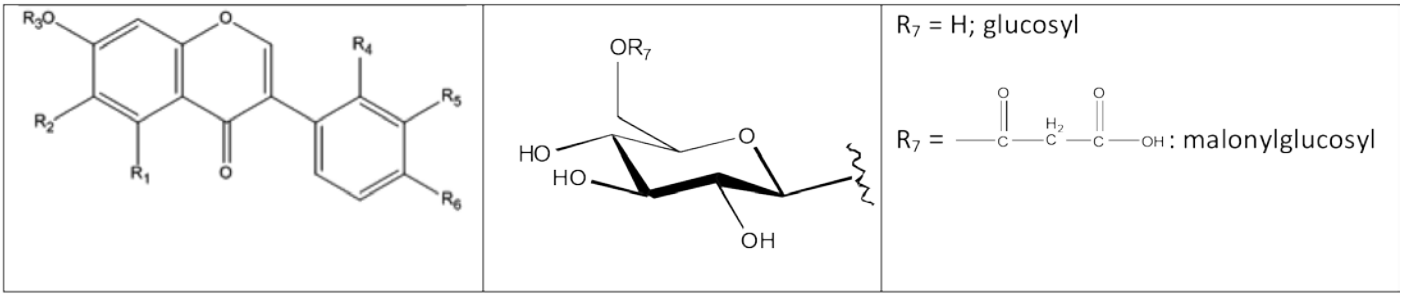
Phenolic acids are a group of phenolic compounds known to have strong antioxidant activity since these acids can scavenge free radicals and neutralize reactive oxygen species (ROS) (Kim et al. 2006; Razzaghi-Asl et al. 2013; Rice-Evans et al. 1996). In the leaves and flowers of red clover, phenolic acids such as protocatechuic acid, *p*-hydroxybenzoic acid, gentisic acid, caffeic acid, *p*-coumaric acid, ferulic acid, and salicylic acid have been identified (Kicel and Wolbis 2006; Kolodziejczyk-Czepas 2012). The overall concentration of phenolic acids is higher in flowers; conversely caffeic and salicylic acids are higher in leaves (Kicel and Wolbis 2006). In another report, concentrations of caffeic acid in leaf extracts of red clover plants exposed to UV-B increased by 40% (Swinny and Ryan 2005). A conjugated form of caffeic acid known as phaseolic acid has also been previously reported in red clover plants (Yoshihara et al. 1977).

Red clover leaves can accumulate approximately 5 µmol/g fresh weight of phaseolic acid (Sullivan 2009). Phaseolic acid is an *o*-diphenol and can prevent post-harvest storage breakdown of forage protein by self-oxidation to *o*-quinone, which is catalyzed by polyphenol oxidases (Sullivan and Zarnowski 2010).

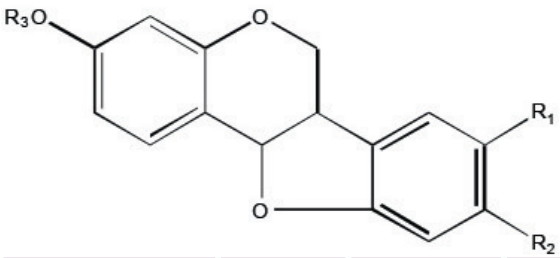
An under-investigated constituent category in red clover are proanthocyanidins, the highest concentration of which occurs in the flowers. Thiolysis data shows red clover proanthocyanidins to be predominantly a procyanidin polymer mixture with an average degree of polymerization (DP) of 9.3. The terminal units consist mainly of catechin (95%) with the remainder made up of epicatechin (5%) (Sivakumaran et al. 2004).

Figure 6i Clinically tested powdered red clover extract *(Linnea SA Riazzino, Switzerland)*

Figure 7a **Isoflavone aglycones, glycosides, and glycoside malonate derivatives in red clover**

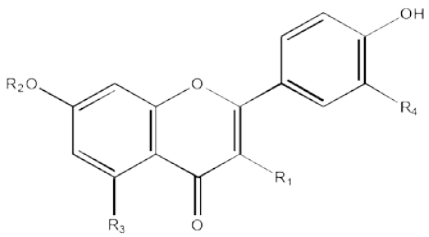


Isoflavones	R_1	R_2	R_3	R_4	R_5	R_6
Aglycones						
Formononetin	H	H	H	H	H	OCH_3
Biochanin A	OH	H	H	H	H	OCH_3
Glycitein	H	OCH_3	H	H	H	OH
Pratensein	OH	H	H	H	OH	OCH_3
Prunetin	OH	H	CH_3	H	H	OH
Genistein	OH	H	H	H	H	OH
Daidzein	H	H	H	H	H	OH
Orobol	OH	H	H	H	OH	OH
Pseudobaptigenin	H	H	H	H	-O-	$-OCH_2-$
Irilone	OH	-O-	$-CH_2-$	H	H	OH
Calycosin	H	H	H	H	OH	OCH_3
Glycosides						
Daidzin	H	H	Glucosyl	H	H	OH
Glycitin	H	OCH_3	Glucosyl	H	H	OH
Genistin	OH	H	Glucosyl	H	H	OH
Pseudobaptigenin-7-*O*-β-D-glucoside	H	H	Glucosyl	H	-O-	$-OCH_2-$
Ononin	H	H	Glucosyl	H	H	OCH_3
Irilone-4′-*O*-β-D-glucoside	OH	-O-	$-CH_2-$	H	H	Glucosyl
Prunetin-4′-*O*-β-D-glucoside	OH	H	CH_3	H	H	Glucosyl
Biochanin A-7-*O*-β-D-glucoside	OH	H	Glucosyl	H	H	OCH_3
Glycoside Malonates						
Daidzin-6″-*O*-malonate	H	H	Malonylglucosyl	H	H	OH
Genistin-6″-*O*-malonate	OH	H	Malonylglucosyl	H	H	OH
Orobol-7-*O*-β-D-glucoside-6″-*O*-malonate	OH	H	Malonylglucosyl	H	OH	OH
3′-methylorobol-7-*O*-β-D-glucoside-6″-*O*-malonate	OH	H	Malonylglucosyl	H	CH_3	OH
Calycosin-7-*O*-β-D-glucoside-6″-*O*-malonate	H	H	Malonylglucosyl	H	OH	OCH_3
Pratensein-7-*O*-β-D-glucoside-6″-*O*-malonate	OH	H	Malonylglucosyl	H	OH	OCH_3
Pseudobaptigenin-7-*O*-β-D-glucoside-6″-*O*-malonate	H	H	Malonylglucosyl	H	-O-	$-OCH_2-$
Formononetin-7-*O*-β-D-glucoside-6″-*O*-malonate	H	H	Malonylglucosyl	H	H	OCH_3
Irilone-4′-*O*-β-D-glucoside-6″-*O*-malonate	OH	-O-	$-CH_2-$	H	H	Malonylglucosyl
Afrormosin-7-*O*-β-D-glucoside-6″-*O*-malonate	H	OCH_3	Malonylglucosyl	H	H	OCH_3
Biochanin A-7-*O*-β-D-glucoside-6″-*O*-malonate	OH	H	Malonylglucosyl	H	H	OCH_3
Texasin-7-*O*-β-D-glucoside-6″-*O*-malonate	H	OH	Malonylglucosyl	H	H	OCH_3
5,7,2′-trihydroxy-6-methoxyisoflavone-7-*O*-β-D-glucoside-6″-*O*-malonate	OH	OCH_3	Malonylglucosyl	OH	H	OH
Prunetin-4′-*O*-β-D-glucoside-6″-*O*-malonate	OH	H	CH_3	H	H	Malonylglucosyl



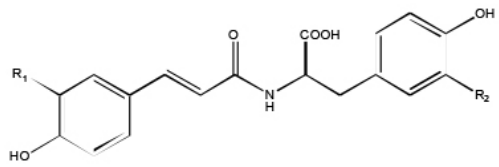
Pterocarpans	R_1	R_2	R_3
Trifolirhizin	-O-	$-OCH_2-$	Glucosyl
Maackiain	-O-	$-OCH_2-$	H
Medicarpin	H	OCH_3	H

Figure 7b Pterocarpans



Flavone	R1	R2	R3	R4
Quercetin	OH	H	OH	OH
Isoquercitrin-6″-*O*-malonate	Malonylglucosyl	H	OH	OH
3-methylquercetin-7-O-β-D-glucoside	OCH_3	Glucosyl	OH	OH

Figure 7c Flavones



Flavone	R1	R2
Clovamide	OH	OH
Caffeoyltyrosine	OH	H
ρ-coumaroyl DOPA	H	OH
ρ-coumaroyltyrosine	H	H

Figure 7d Phenolic Compounds

Clovamides

Clovamides are polyphenolic amides, belonging to the class of hydroxycinnamic acid amides linked to aromatic amino acids. Clovamides were identified for the first time in red clover by Yoshihara et al. (1974) and named after the plant of origin. This class of compounds is found in relatively few plants and is most abundant in clovers. In red clover, caffeoyl DOPA (clovamide) is the most abundant, while other amides such as caffeoyltyrosine, *p*-coumaroyl DOPA, and *p*-coumaroyltyrosine are found in trace quantities. These compounds are more abundant in the shoots of the plant compared to the roots (Lin et al. 2000; Tebayashi et al. 2000). According to Sullivan and Zarnowski 2011, fresh red clover leaves accumulate up to several mmol/kg^{-1} of clovamide. Clovamide exhibits in vitro neuronal protective effects at concentrations of 10–100 µmol·L^{-1} with EC_{50} values of (0.9–3.7 µmol·L^{-1}). In this study, the maximal effects ranged from 40% to 60% protection from cell death over untreated controls at 100 µmol·L^{-1} (Fallarini et al. 2011).

Essential Oil

Volatile organic compounds contained in the essential oil fraction are secondary metabolites that are either inherent in red clover or are induced by damage or stress. The volatile compounds in fresh red clover leaf primarily exist as alcohols (15.6%), ketones (15.7%), and terpenes (24.5%). Other notable classes of compounds include alkanes (3.70%), aldehydes (5.38%), and esters (1.20%) (Figueiredo et al. 2007). Several of the most abundant individual compounds in red clover have been identified and quantified in terms of their relative percent composition and include: 3-octanol (3.44%), 6,10,14-trimethyl-2-pentadecanone (3.46%), benzaldehyde (3.74%), (Z)-β-caryophyllene (4.05%), β-farnesene (4.86%), 3-methyl-1-butanol (5.73%), and 3-octanone co-eluting with 6-methyl-5-hepten-2-one (8.60%) (Figueiredo et al. 2007).

Volatile components associated with different parts of red clover have been described in leaves with widely varying concentrations reported, primarily due to differences in analytical methodologies. For example, the following compounds and their values have been reported. **Leaves:** (Z)-3-Hexenyl acetate (33%), (Z)-3-hexenol (24%), and (E)- and (Z)-β-ocimenes (22% and 5%, respectively) (Buttery et al. 1984); **Flowers:** Acetophenone (24%), methyl cinnamate (11%), and 1-phenylethanol (8%). **Seed pods:** (E)- and (Z)-β-ocimenes (35% and 6%, respectively) (Buchbauer et al. 1996; Buttery et al. 1984). Volatiles in red clover root extracts include (E)-2-hexenal, benzaldehyde, octanoic acid, and decanal at concentrations of 2.12, 0.44, 0.43, and 0.50 µg/mL, respectively (Tapia et al. 2007).

In more recent analytical work, Cecotti et al. 2013, reported a total steam-distilled oil content of 0.006 to 0.011% in fresh plant material of *T. pratense* ssp. Nivale. The primary compounds identified were alcohols, aldehydes, hydrocarbons, terpenes, phenolics, ketones, acids, and esters. The two predominant constituents in the vegetative and flowering phases were oct-1-en-3-ol and phenacetylaldehyde and oil concentration was greater in leaves than

flowers, suggesting these compounds are to protect against predation. The linear alcohol Z-3-hexanol also occurred in relative abundance at 3.1 ± 0.5% to 7.4 ± 0.4% (fresh weight).

Interestingly, the aroma of essential oil of red clover is described as "lovage-like" (Buchbauer et al. 1996).

ANALYTICAL

High Performance Thin Layer Chromatography (HPTLC) for the identification of Red Clover (*Trifolium pratense*)

Apolar Isoflavones

Sample Preparation

1 g powdered raw material, 100 mg dry extract, or corresponding amount of tablet is mixed with 10 mL methanol-water (6:4); sonicate for 10 min and centrifuge. The supernatant is used as the test solution.

Standard Preparation (optional)

1 mg formononetin is dissolved in 5 mL methanol.
1 mg each biochanin A, daidzein, and genistein are dissolved in 1 mL methanol each.

Reagent Preparation

Natural Products reagent (NP reagent): 1 g diphenylboric acid aminoethylester is dissolved in 200 mL ethyl acetate.

Chromatographic Conditions

Stationary Phase:
HPTLC plates 10 x 10 cm or 20 x 10 cm silica gel 60 F 254.

Mobile Phase:
Toluene, ethyl acetate, formic acid (70:30:1).

Sample Application:
4 µL of test solution and 2 µL of standard are applied each as 8-mm bands with a minimum distance of 2 mm between bands. Application position should be 8 mm from lower edge of plate.

Development:
10 x 10 cm or 20 x 10 cm Twin Trough Chamber, saturated for 20 min with filter paper, 5 or 10 mL developing solvent in each trough. Developing distance is 70 mm from lower edge of plate. Dry plate in a stream of warm air for five minutes.

Detection:
a) UV 254 nm.
b) The plate is heated at 100 °C for three min, dipped while still hot in NP reagent, then dried in a stream of cold air. Examination under UV 366 nm.

Results:
Compare to the chromatograms provided.

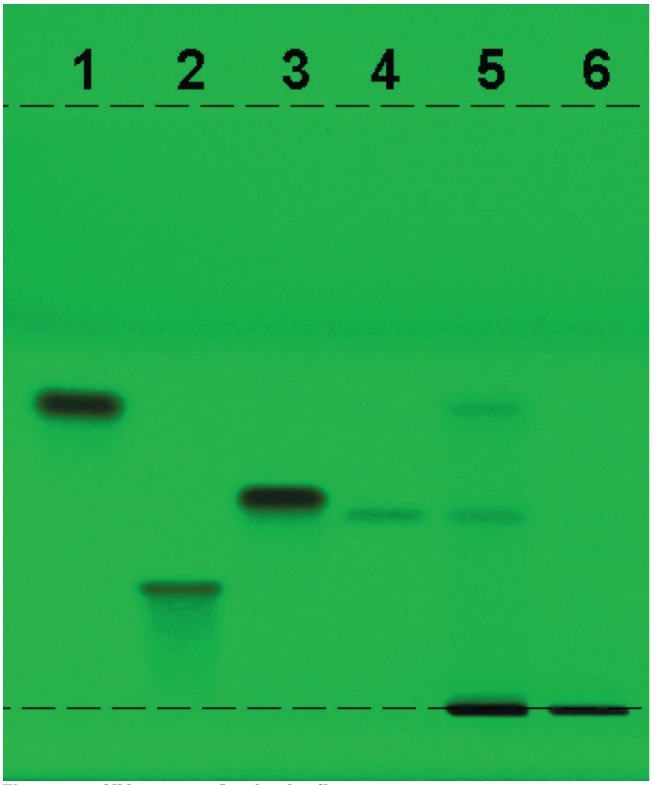


Figure 8a UV 254 nm: Apolar isoflavones

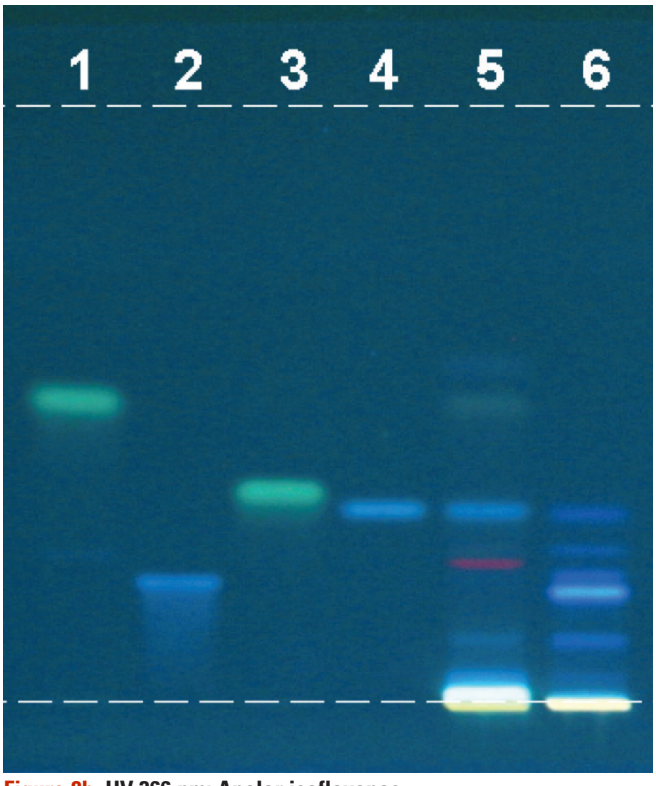


Figure 8b UV 366 nm: Apolar isoflavones

Lane 1 Biochanin A
Lane 2 Daidzein
Lane 3 Genistein
Lane 4 Formononetin
Lane 5 Red clover blossoms (organically cultivated)
Lane 6 White clover (*Trifolium repens* wildcrafted, Switzerland)

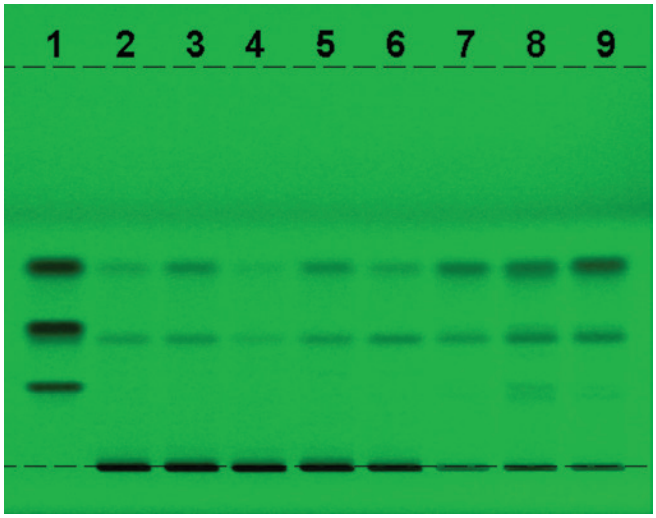


Figure 8c **UV 254 nm: Apolar isoflavones**

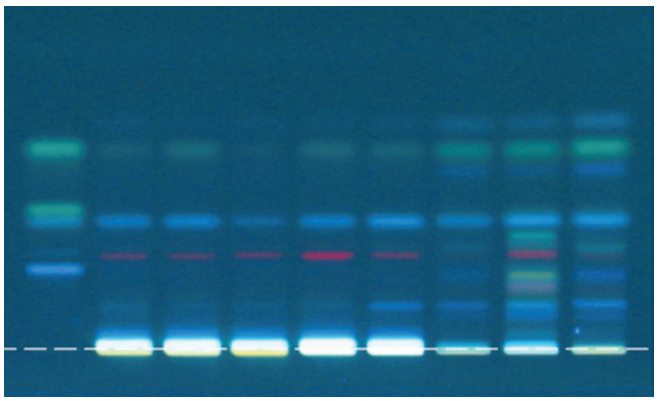


Figure 8d **UV 366 nm: Apolar isoflavones**

Lane 1	Daidzein, formononetin, genistein, biochanin A (increasing Rf)
Lane 2	Red clover blossoms
Lane 3	Red clover blossoms
Lane 4	Red clover blossoms
Lane 5	Red clover aerial with blossoms
Lane 6	Red clover leaves and stem
Lane 7	Red clover tablet (clinically tested extract)
Lane 8	Red clover tablet
Lane 9	Red clover extract (USP)

Discussion of the chromatograms

Figure 8a (UV 254 nm apolar isoflavones): All standards show a dark band. Biochanin A on Lane 1 at Rf ~0.5, daidzein on Lane 2 at Rf ~0.2, genistein on Lane 3 at Rf ~0.35, and formononetin on Lane 4 at Rf ~0.33. There are bands in the red clover sample on Lane 5 corresponding to biochanin A and formononetin. The white clover sample shows no zones with this visualization.

Figure 8b (UV 366 nm apolar isoflavones): All standards on Lanes 1–4 show fluorescing green or blue bands. In the red clover sample (Lane 5) there is a light green band corresponding to biochanin A (Rf 0.5) and a light blue band corresponding to formononetin (Rf 0.33). An additional faint blue band is present in the lower Rf region and another red band just below the blue band corresponding to formononetin. An additional light green band appears immediately above the green band corresponding to biochanin A. The white clover sample shows many blue fluorescing zones, three of them at the approximate positions of daidzein, genistein, and formononetin standards. However, these zones cannot be assigned as daidzein or formononetin because they are not seen under UV 254 nm.

Figure 8c (UV 254 nm apolar isoflavones and red clover preparations): All standards on Lane 1 show a dark band: daidzein at Rf ~0.2, formononetin at Rf ~0.33 (faint band), genistein at Rf ~0.35, and biochanin A at Rf ~0.5. There are bands of varying intensity in all red clover samples on Lanes 2–9 corresponding to biochanin A and formononetin. The red clover tablet and extract on Lanes 8 and 9 additionally show very weak bands corresponding to daidzein and genistein.

Figure 8d (UV 366 nm apolar isoflavones and red clover preparations): All standards on Lane 1 show fluorescing bands. Daidzein (Rf ~0.2) and formononetin (Rf ~0.33) show blue fluorescent bands, while those for genistein (Rf ~0.35) and biochanin A (Rf ~0.5) are green. There are bands of varying intensities in all red clover samples on Lanes 2–9 corresponding to biochanin A and formononetin. Daidzein and genistein are difficult to assign due to the low concentration and overlapping of the bands in the samples. All samples also show a weak blue fluorescing zone above biochanin A. The tablet and extract samples (Lanes 7–9) show a blue fluorescing zone below biochanin A and additional zones in the lower Rf region. The red zone seen in most of the samples is probably due to chlorophyll. The leaf and stem sample (Lane 6) shows a stronger blue band at RF ~0.15 than in the other raw material samples. This is also present in the extracts (Lanes 7–9).

Note: The concentration of daidzein and genistein in red clover is much lower than formononetin and biochanin A. Daidzein and genistein are likely present in the sample in amounts lower than the HPTLC detection limit.

Polar Isoflavones and Isoflavone Glycosides

Sample preparation

1 g powdered raw material, 100 mg dry extract or corresponding amount of tablet is mixed with 10 mL methanol-water (6:4); sonicate for 10 min and centrifuge. The supernatant is used as the test solution.

Standard preparation (optional)

1 mg each rutin, hyperoside, and isoquercitrin are dissolved in 10 mL methanol each.
1 mg each daidzin and genistin are dissolved in 1 mL methanol each.

Reagent preparation

Natural Products (NP) reagent: 1 g diphenylboric acid aminoethylester is dissolved in 200 mL ethyl acetate.

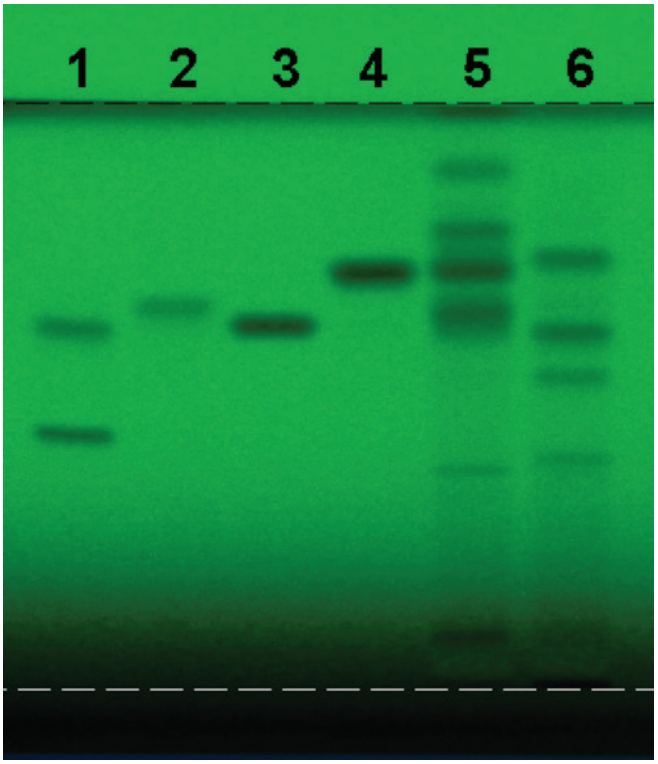


Figure 9a **UV 254 nm polar isoflavones**

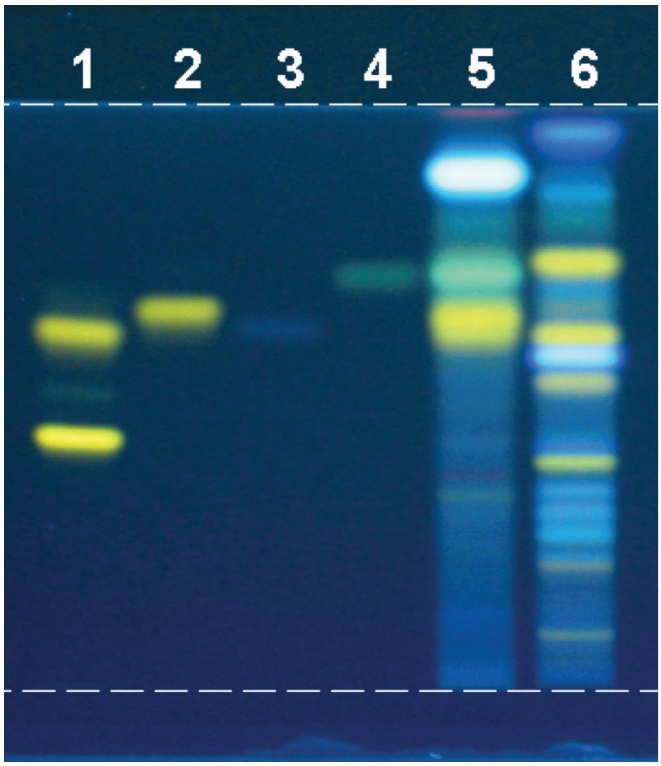


Figure 9b **UV 366 nm polar isoflavones**

Lane 1 Rutin and hyperoside (increasing Rf)
Lane 2 Isoquercitrin
Lane 3 Daidzin
Lane 4 Genistin
Lane 5 Red clover blossoms
Lane 6 White clover, Switzerland, wild-crafted

Chromatographic Conditions

Stationary Phase:

HPTLC plates 10 x 10 cm or 20 x 10 cm silica gel 60 F 254.

Mobile Phase:

Ethyl acetate, formic acid, acetic acid, water (100:11:11:27).

Sample Application:

For crude botanical material, apply 2 µL of the test solution. For extracts, greater concentrations need to be applied depending on the strength of the extract (approximately 20 µL). Apply 2 µL of standard each as an 8-mm band with a minimum distance of 2 mm between bands. Application position should be 8 mm from the lower edge of the plate.

Development:

10 x 10 cm or 20 x 10 cm Twin Trough Chamber, saturated for 20 min with filter paper, 5 or 10 mL developing solvent in each trough. Developing distance is 70 mm from lower edge of plate. Dry plate in a stream of warm air for five minutes.

Detection:

a) UV 254 nm
b) The plate is heated at 100 °C for three min, dipped while still hot in NP reagent, then dried in a stream of cold air. Examination under UV 366 nm.

Results:

Compare to the chromatograms provided.

Discussion of the chromatograms

Figure 9a (UV 254 nm polar isoflavones): All standards show a dark band. On Lane 1, rutin occurs at Rf~0.5 and hyperoside at Rf ~0.7. On Lane 2, isoquercitrin occurs at Rf ~0.75. On Lane 3, daidzin occurs at Rf ~0.7. On Lane 4 genistin occurs at Rf ~0.8. There is a band in the red clover sample on Lane 5 at the position of genistin, however, this is not genistin, and a cluster of zones at the position of hyperoside/isoquercitrin/daidzin. Additional dark bands are seen. The white clover sample shows a band at the position of hyperoside/daidzin.

Figure 9b (UV 366 nm polar isoflavones): All standards show fluorescing bands of various color (yellow, blue, green). Rutin (Rf ~0.5) and hyperoside (Rf ~0.7) (both on Lane 1) and isoquercitrin (Rf ~0.75) (Lane 2) show as yellow zones. Daidzin shows as a faint blue band at Rf ~0.7 (Lane 3). Genistin (Lane 4), shows as a green band at Rf ~0.8. Red clover (Lane 5) is characterized by the presence of two prominent bright yellow bands at the level of hyperoside and isoquercetin, a bluish-green band at the level of genistin, and a prominent bluish-white band in the upper Rf region. Other faint bands occur at in the lower to middle

Rf range. If present, daidzin could not be detected (overlapping). The white clover sample is clearly distinguished from red clover. White clover shows two brown bands in the lower Rf region; a cluster of three well-defined blue bands in the lower to middle Rf region; a yellow band occurring below the level of rutin; a grouping of a yellow, blue, and yellow band around the level of hyperoside; another yellow band above the level of genistin; and three additional greenish-blue and blue bands in the upper Rf region.

Figure 9c (UV 254 nm polar isoflavones): All standards on Lane 1 show as dark bands: rutin at Rf~0.5, hyperoside and daidzin overlapping at Rf~0.7, isoquercitrin at Rf~0.75, and genistin at Rf~0.8. There is a band in the red clover samples on Lanes 2–6 corresponding to genistin and a cluster of zones at the position of hyperoside/isoquercitrin/daidzin. Additional dark bands are seen. The tablet sample on Lane 7 and the pharmacopoeial extract sample on Lane 9 are characterized by three prominent dark bands between Rf ~0.7–0.8. No clear zones are observed in the extract sample on Lane 7.

Figure 9d (UV 366 nm polar isoflavones): All standards show fluorescing yellow and greenish bands of (daidzin is not seen due to interference with hyperoside). The red clover samples (Lanes 2–6) are characterized by faint bands of varying color in the lower to middle Rf region; a prominent yellow band at the level of genistin; a prominent greenish-blue band above the level of genistin and another bright bluish-white band in the upper Rf region. Zones corresponding to hyperoside and isoquercitrin can be detected in samples on Lanes 2–7 and 9. These zones are very faint in the sample on Lane 8. If present, a band corresponding to daidzin could not be detected (overlapping). A faint band corresponding to rutin is seen in samples on Lanes 3–5.

Note: The concentration of daidzin and genistin found in red clover is very low. Daidzin and genistin are likely present in the sample in amounts lower than the HPTLC detection limit.

High Performance Liquid Chromatography for Red Clover Aerial Parts and Blossoms, Powder, Dry Extract, and Aglycones Dry Extract—Identification and Quantitation

The primary analytes of interest for quantification in red clover herb and extracts are isoflavones. The United Sates Pharmacopeial Convention (USP) includes monographs for red clover aerial parts, powdered red clover, powdered red clover extract, red clover isoflavone aglycones dry extract, and red clover tablets*, providing analytical methodologies and specifications for raw herb, extracts, and finished dosage forms. Chromatographic methodology and specifications featured in USP parallel those developed for the commercial extract employed in the majority of clinical trials (Promensil®), that typically contains up to 40% of

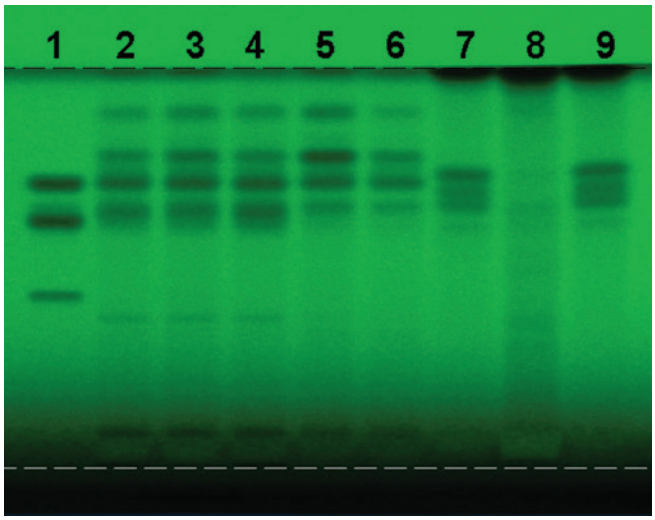


Figure 9c UV 254: Polar isoflavones

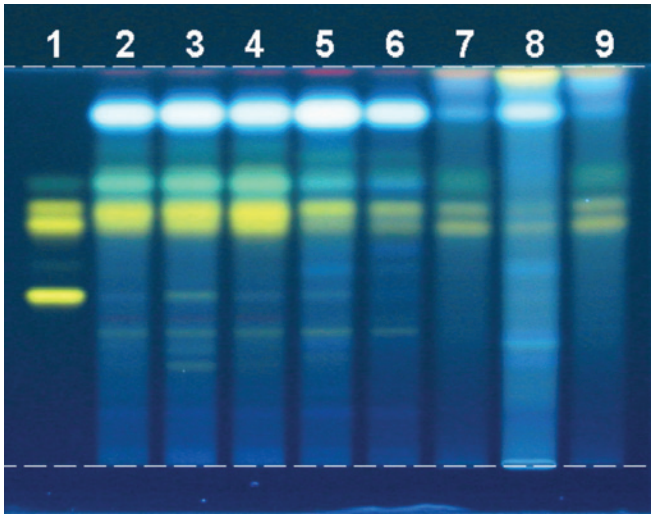


Figure 9d UV 366: Polar isoflavones

Lane 1	Rutin, hyperoside/daidzin, isoquercitrin, genistin (increasing Rf)
Lane 2	Red clover blossoms
Lane 3	Red clover blossoms
Lane 4	Red clover blossoms
Lane 5	Red clover blossoms
Lane 6	Red clover leaves and stem
Lane 7	Red clover tablet (clinically tested)
Lane 8	Red clover tablet
Lane 9	Red clover extract (USP)

isoflavone aglycones with a ratio of 5,7-dihydroxyisolflavones to 7-hydroxyisoflavones between 0.9 and 1.7. Red clover extracts for which aglycones are not claimed, can employ analogous analytical scheme but should incorporate the acid hydrolysis step in the sample work-up. Such extracts may or may not conform to the same isoflavone ratio.

Chromatographic analysis of red clover isoflavones is conventionally conducted following their conversion to aglycones. The intact raw herb is subjected to acid-catalyzed hydrolytic cleavage, whereas the aglycone extract already contains isoflavones as aglycones and thus does not require hydrolysis. The USP analytical methods are included here

with permission of USP (USP-NF 2016).

* Tablets can be difficult to analyze due to the relatively small amounts of red clover they typically contain.

Identification of Red Clover Raw Herb and Powdered Red Clover

Solvent: Ethanol and water (1:1)
Solution A: Acetonitrile and water (1:3) containing 0.05% trifluoroacetic acid
Solution B: Acetonitrile containing 0.05% trifluoroacetic acid

Mobile Phase

Time (min)	Solution A (%)	Solution B (%)
0	100	0
2	100	0
2.5	87	13
7.5	80	20
7.8	73	27
8.0	55	45
11.0	50	50
13.0	40	60
15.0	26	74
16.0	0	100
18.1	100	0
23.0	100	0

Standard stock solution: Transfer a quantity of USP Powdered Red Clover Extract RS, equivalent to 30 mg of the labeled content of isoflavones, to a 250-mL volumetric flask. Add 15 mL of anhydrous ethanol, sonicate until dissolved, and dilute with *Solvent* to volume.

Standard solution: Evaporate 50 mL of *Standard stock solution* to dryness under vacuum. Add 15 mL of 2 N hydrochloric acid and heat on a water bath for 30 min. Transfer the resulting solution, with the aid of 15 mL of ethanol, to a 50-mL volumetric flask, and dilute with *Solvent* to volume. Centrifuge, or filter through a 0.45-µm PVDF membrane.

Sample stock solution: Transfer 2.5 g of ground plant material, accurately weighed, into a 120-mL flask with a stopper. Add exactly 100 mL of *Solvent*, close the flask, and shake on an orbital or wrist-action shaker for at least 12 h.

Sample solution: Evaporate 50 mL of *Sample stock solution* to dryness under vacuum at 40 °C. Add 15 mL of 2 N hydrochloric acid, and heat on a water bath for 30 min. Quantitatively transfer this solution, with the aid of 15 mL of ethanol, to a 50-mL volumetric flask, and dilute with *Solvent* to volume. Filter through a 0.45-µm PVDF membrane, discarding the first 4 mL of the filtrate.

Chromatographic Conditions

Apparatus:	LC (see USP Chromatography <621>, System Suitability)
Detection:	UV 254 nm
Column:	4.6 mm × 25 cm; end-capped 5-µm (e.g., Waters® Symmetry)
Column temperature:	45 °C
Flow rate:	1.0 mL/min
Injection volume:	10 µL

System suitability requirements
Chromatogram similarity
The chromatogram of *Standard solution* is similar to the reference chromatogram provided with the lot of the reference USP Powdered Red Clover Extract RS used.

Tailing factor
NMT 2.0 for the formononetin peak

Analysis
Identify the peaks corresponding to daidzein, genistein, formononetin, and biochanin A in the *Sample solution* chromatogram by comparison with the chromatogram obtained with the *Standard solution*. Measure the analyte peak areas.

$$\text{Result} = (B + G)/(D + F)$$

B = area of biochanin A
G = area of genistein
D = area of daidzein
F = area of formononetin

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks for daidzein, genistein, formononetin, and biochanin A at retention times that correspond to those in the chromatogram of *Standard solution* and the ratio of 5,7- dihydroxyisoflavones to 7-hydroxyisoflavones is between 0.1 and 10.

Identification of Red Clover Dry Extract

The solvent, mobile phase, chromatographic conditions, standard solutions preparation, analysis, and acceptance criteria are the same as in ***Identification of Red Clover Raw Herb and Powdered Red Clover*** above with exception of the *Sample solution*, which is modified as follows:

Sample solution: Transfer a quantity of Red Clover Dry Extract equivalent to 6 mg of the labeled content of isoflavones to a 50-mL volumetric flask. Add 15 mL of 2N hydrochloric acid, sonicate to disperse, and heat on a water bath for 30 min. Allow to cool, add 15 mL of ethanol, and dilute with *Solvent* to volume. Centrifuge, or filter through a 0.45-µm PVDF membrane, discarding the first 4 mL of the filtrate.

Identification of Red Clover Isoflavone Aglycones Dry Extract

The solvent, mobile phase, chromatographic conditions and

analysis are the same as in ***Identification of Red Clover Raw Herb and Powdered Red Clover*** above.

Standard solution: Prepare a 0.10 - 0.15 mg/mL solution of USP Red Clover Aerial Parts Isoflavone Aglycones Dry Extract in *Solvent*, sonicate briefly, and filter through a 0.45-µm PVDF membrane.

Sample solution: Prepare a 0.12 mg/mL solution of red clover isoflavone aglycones dry extract in *Solvent*, sonicate briefly, and filter through a 0.45-µm PVDF membrane.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks for daidzein, genistein, formononetin, and biochanin A at retention times that correspond to those in the chromatogram of *Standard solution* and the ratio of 5,7- dihydroxyisoflavones to 7-hydroxyisoflavones is between 0.9 and 10.7.

Content of Isoflavones in Red Clover Raw Herb and Powdered Red Clover

The solvent, mobile phase and chromatographic conditions are the same as in ***Identification of Red Clover Raw Herb and Powdered Red Clover*** above.

Standard stock solution A: Transfer a quantity of USP Powdered Red Clover Extract RS, equivalent to 30 mg of the labeled content of isoflavones, to a 250-mL volumetric flask. Add 15 mL of anhydrous ethanol, sonicate until dissolved, and dilute with *Solvent* to volume.

Standard solution A: Evaporate 50 mL of *Standard stock solution* A to dryness under vacuum. Add 15 mL of 2 N hydrochloric acid, and heat on a water bath for 30 min. Transfer the resulting solution, with the aid of 15 mL of ethanol, to a 50-mL volumetric flask, and dilute with *Solvent* to volume. Centrifuge, or filter through a 0.45-µm PVDF membrane.

Standard solution B: 0.1 mg/mL of USP Formononetin RS, 0.02 mg/mL of USP Genistein RS, 0.02 mg/mL of USP Daidzein RS, and 0.1 mg/mL of USP Biochanin A RS in a mixture of n-propanol and water (1:1). Sonicate, and filter through a 0.45-µm PVDF membrane.

Sample stock solution: Transfer 2.5 g of ground plant material, accurately weighed, into a 120-mL flask with a stopper. Add exactly 100 mL of Solvent, close the flask, and shake on an orbital or wrist-action shaker for at least 12 h.

Sample solution: Evaporate 50 mL of *Sample stock solution* to dryness under vacuum at 40 °C. Add 15 mL of 2 N hydrochloric acid, and heat on a water bath for 30 min. Quantitatively transfer this solution, with the aid of 15 mL of ethanol, to a 50-mL volumetric flask, and dilute with *Solvent* to volume. Filter through a 0.45-µm PVDF membrane, discarding the first 4 mL of the filtrate.

Standard Solution Stability: One year in the refrigerator at 6 ± 4 °C

Storage of Reference Standards: Freezer at -25 ± 3 °C

System suitability
Samples: *Standard solution A* and *Standard solution B*

Suitability requirements
Chromatogram similarity
The chromatogram of *Standard solution A* is similar to the reference chromatogram provided with the lot of the reference USP Powdered Red Clover Extract RS used.

Tailing factor
NMT 2.0 for the formononetin peak, *Standard solution B*.

Relative standard deviation
NMT 2.0%, for the formononetin peak in replicate injections, *Standard solution B*.

Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Identify the peaks corresponding to daidzein, genistein, formononetin, and biochanin A in the *Sample solution* chromatogram by comparison with the chromatogram obtained from *Standard solution A* and the reference chromatogram. Measure the areas of the analyte peaks. Separately calculate the percentages of daidzein, genistein, formononetin, and biochanin A:

$$\text{Result} = (r_u / r_s) \times C_s \times (V/W) \times D \times 100$$

r_u = Peak area of the relevant isoflavone in the *Sample solution*
r_s = Peak area of the corresponding isoflavone in the *Standard solution B* (daidzein, genistein, formononetin, or biochanin A)
C_s = Concentration of daidzein, genistein, formonone tin, or biochanin A in the *Standard solution B* (mg/mL)
V = Volume of the *Sample stock solution* (mL)
W = Weight of red clover used to prepare the *Sample solution* (mg)
D = Dilution factor to prepare the *Sample solution* from the *Sample stock solution*

Acceptance criteria: The sum percentages of daidzein, genistein, formononetin, and biochanin A is not less than 0.5% (w/w) on the dry weight basis.

Content of Isoflavones in Red Clover Dry Extract

Definition
Red clover dry extract (USP) is prepared by extraction of

the extract with hydroalcoholic mixtures or other suitable solvents. The ratio of plant material to extract is 3:1–25:1. It contains not less than 90.0% and not more that 110.0% of the labeled amount of isoflavones, calculated on the dried basis as the sum of daidzein, genistein, formononetin, and biochanin A. It may contain suitable added substances.

The solvent, mobile phase and chromatographic conditions are the same as in ***Identification of Red Clover Raw Herb and Powdered Red Clover*** above. The standard solutions are prepared as in ***Content of Isoflavones in Red Clover Raw Herb and Powdered Red Clover*** above.

Sample solution: Accurately transfer a quantity of red clover dry extract equivalent to 6 mg of the labeled content of isoflavones to a 50-mL volumetric flask. Add 15 mL of 2N hydrochloric acid, sonicate to disperse, and heat on a water bath for 30 min. Allow to cool, add 15 mL of ethanol, and dilute with *Solvent* to volume. Centrifuge, or filter through a 0.45-µm PVDF membrane, discarding the first 4 mL of the filtrate.

Analysis

Samples: *Standard solution* A, *Standard solution B*, and *Sample solution*

Identify the peaks corresponding to daidzein, genistein, formononetin, and biochanin A in the *Sample solution* chromatogram by comparison with the chromatogram obtained from *Standard solution* A and the reference chromatogram. Measure the areas of the analyte peaks. Separately calculate the percentages of daidzein, genistein, formononetin, and biochanin A:

$$\text{Result} = (r_u / r_s) \times C_s \times (V/W) \times 100$$

r_u = Peak area of the relevant isoflavone in the *Sample solution*

r_s = Peak area of the corresponding isoflavone in the *Standard solution B* (daidzein, genistein, formononetin, or biochanin A)

C_s = Concentration of daidzein, genistein, formonone tin, or biochanin A in *Standard solution B* (mg/mL)

V = Volume of the *Sample solution* (mL)

W = Weight of the red clover dry extract used to prepare the *Sample solution* (mg)

$$\text{Result} = \Sigma Pi/L \times 100$$

ΣPi = Total combined content of isoflavones as determined above

L = Labeled amount of isoflavones

Acceptance criteria: 90.0%–110.0% of the labeled amount of isoflavones as the sum of daidzein, genistein, formononetin, and biochanin A, on a dry weight basis.

Content of Isoflavones in Red Clover Isoflavone Aglycones Dry Extract

Definition

Red Clover Isoflavone Aglycones Dry Extract is prepared by extraction with hydroalcoholic mixtures or other suitable solvents. It contains not less than 36.0% and not more that 44.0% of isoflavone aglycones, calculated on the dried basis as the sum of daidzein, genistein, formononetin, and biochanin A. It contains not more than 1.0% of daidzein, and not more than 1.0% of genistein; these limits facilitate detection of material derived from other isoflavone-rich sources, notably soy. It may contain suitable excipients.

The solvent, mobile phase and chromatographic conditions are the same as in ***Identification of Red Clover Raw Herb and Red Powdered Clover*** above. *The Standard solution B* is prepared as in ***Content of Isoflavones in Red Clover Raw Herb and Powdered Red Clover*** above.

Standard solution A: Prepare a 0.10–0.15 mg/mL solution of USP Red Clover Aerial Parts Isoflavone Aglycones Dry Extract in *Solvent*, sonicate briefly, and filter through a 0.45-µm PVDF membrane.

Sample solution: Accurately weigh a quantity of red clover isoflavone aglycones dry extract equivalent to 12 mg of the labeled content of isoflavones and transfer to a 100-mL volumetric flask. Add 50 mL of *Solvent*, sonicate to disperse, and dilute with *Solvent* to volume. Centrifuge, or filter through a 0.45-µm PVDF membrane, discarding the first 4 mL of the filtrate.

Analysis

Samples: *Standard solution* A, *Standard solution B*, and *Sample solution*

Identify the peaks corresponding to daidzein, genistein, formononetin, and biochanin A in the *Sample solution* chromatogram by comparison with the chromatogram obtained from *Standard solution A* and the reference chromatogram. Measure the areas of the analyte peaks. Separately calculate the percentages of daidzein, genistein, formononetin, and biochanin A:

$$\text{Result} = (r_u / r_s) \times Cs \times (V/W) \times 100$$

r_u = Peak area of the relevant isoflavone in the Sample solution

r_s = Peak area of the corresponding isoflavone in the *Standard solution B* (daidzein, genistein, for mononetin, or biochanin A)

Cs = Concentration of daidzein, genistein, formonone tin, or biochanin A in *Standard solution B* (mg/mL)

V = Volume of the *Sample solution* (mL)

W = Weight of the red clover isoflavone aglycones dry extract used to prepare the *Sample solution* (mg)

Acceptance criteria: 36.0%–44.0% of isoflavones as the sum of daidzein, genistein, formononetin, and biochanin A, on

the dry weight basis. Not more than 1.0% of daidzein and not more than 1.0% genistein, both on the dry weight basis.

Limit Tests

Raw Material

Foreign Organic Matter:	Not more than 2.0% (USP 32-NSF 27).
Total Ash:	Not more than 10.0% (USP 32-NSF 27).
Acid-insoluble Ash:	Not more than 2.0% (USP 32-NSF 27).
Loss on Drying:	Not more than 12.0% (dried at 1 g at 105 °C for two hours) (USP 32-NSF 27).
Water-soluble Extractives:	Not less than 15.0% via cold-extraction method (USP 32-NSF 27).

Dry Extract

Loss on Drying:	Not more than 3% based on 1 g of extract dried at 105 °C for two hours (USP 32-NSF 27).
Residue on Ignition:	Not more than 4% when igniting 1 g of extract.

THERAPEUTICS

Red clover has a long history of therapeutic use. Traditionally, the blossoms and whole plant were regarded as a "blood purifier" used for skin conditions (e.g., eczema, psoriasis), as an expectorant, and for digestive complaints, among a variety of other uses (see History and Traditional Western Herbal Medicine Supplement). Red clover also has figured prominently in herbal anti-cancer formulas that persist today (e.g., Essiac, Hoxsey Formula). However, historically, references to "cancer" primarily referred any slow healing necrotic sores and do not necessarily correlate with cytologically defined cancers. In more recent years, due to the identification of it containing a host of phytoestrogen compounds (isoflavones), investigations have explored the potential role of red clover in the management of menopausal symptoms. The primary red clover preparation (Promensil) used for this purpose is an extract standardized to deliver 40% of aglycones isoflavones at a ratio between 5,7-dihydroxyisolflavones and 7-hydroxyisoflavones of 0.7:1.9, while a few other preparations were used as well (Menoflavon, Melbrosin Int, Vienna, Austria; MenoStabil, Bad Heilbrunner Naturheilmittel GmbH & Co, Germany; among others) (see Tables 6–9). There is mixed evidence regarding efficacy of red clover on menopausal symptoms (hot flashes and bone health) and good evidence supporting the herb's role in reducing postmenopausal risk of cardiovascular disease. Other data provides some support for red clover's traditional uses, while support for other uses is lacking, primarily due to lack of investigation.

Pharmacokinetics

The main isoflavones of red clover (*Trifolium pratense*) are formononetin and biochanin A and their glycosidic conjugates, ononin, and sissotrin, respectively (Booth et al. 2006b; Heinonen et al. 2004). Formononetin and biochanin A are methylated derivatives of daidzein and genistein, two other isoflavones in red clover which are the better known principle isoflavones in soy. Other isoflavones in red clover include prunetin, pratensein, calycosin, and pseudobaptigenin (Heinonen et al. 2004). Pharmacokinetic studies have been conducted on isoflavones derived from red clover. Investigations of isoflavone pharmacokinetics have been carried out for other botanical sources of isoflavones, mainly soy, and provide additional clues as to the fate of these compounds.

Human Studies

Initial pharmacokinetic studies conducted using pure isoflavones derived from soy, plus their respective β-glycosides, provide some context for studies conducted with red clover preparations. These studies indicated that the bioavailability was greater for the isoflavones when they were ingested as glycosides (daidzin, genistin), rather than aglycones (daidzein and genistein). However the aglycones were absorbed more rapidly than the glucosides. Pharmacokinetic studies were conducted with a total of 19 healthy women, who were divided into four groups. Each group consumed pure daidzein, pure genistein, or their respective β-glycosides) administered as a single bolus dose of 50 mg each (Setchell et al. 2001). Blood samples were obtained up to 24 hours after ingestion of the compounds. The data showed that the mean time to peak plasma concentration (T_{max}) varied between the aglycones and glycosides. The T_{max} for the aglycones was in the range of four and seven hours and that for the glucosides was eight to 11 hours. This time difference is consistent with the time required to cleave the glycoside before absorption of the aglycone by the gut. There were also differences between individual isoflavones, with peak plasma concentrations of genistein being higher than daidzein following administration of equal amounts of each. This difference is accounted for by a more extensive distribution (ratio of dose to plasma concentration) for daidzein (236 L) compared to genistein (161 L). Systematic bioavailability, measured as the plasma drug concentration-time curve (AUC), was 4.54 mL • h for genistein compared to 2.94 mL • h for daidzein. The AUC for genistin was 4.95 mL • h and that for daidzin was 4.52 mL • h.

Pharmacokinetic Data From Red Clover Studies

Setchell et al. (2001) studied the pharmacokinetics of a single bolus of red clover product containing 40 mg total isoflavones (Promensil) given to a single healthy male. The product contained predominately formononetin and biochanin A in the aglycone form with minor amounts of daidzein and genistein. HPLC analysis of blood samples found a rapid increase in plasma concentrations of daidzein and genistein, accounting for more than 95% of the

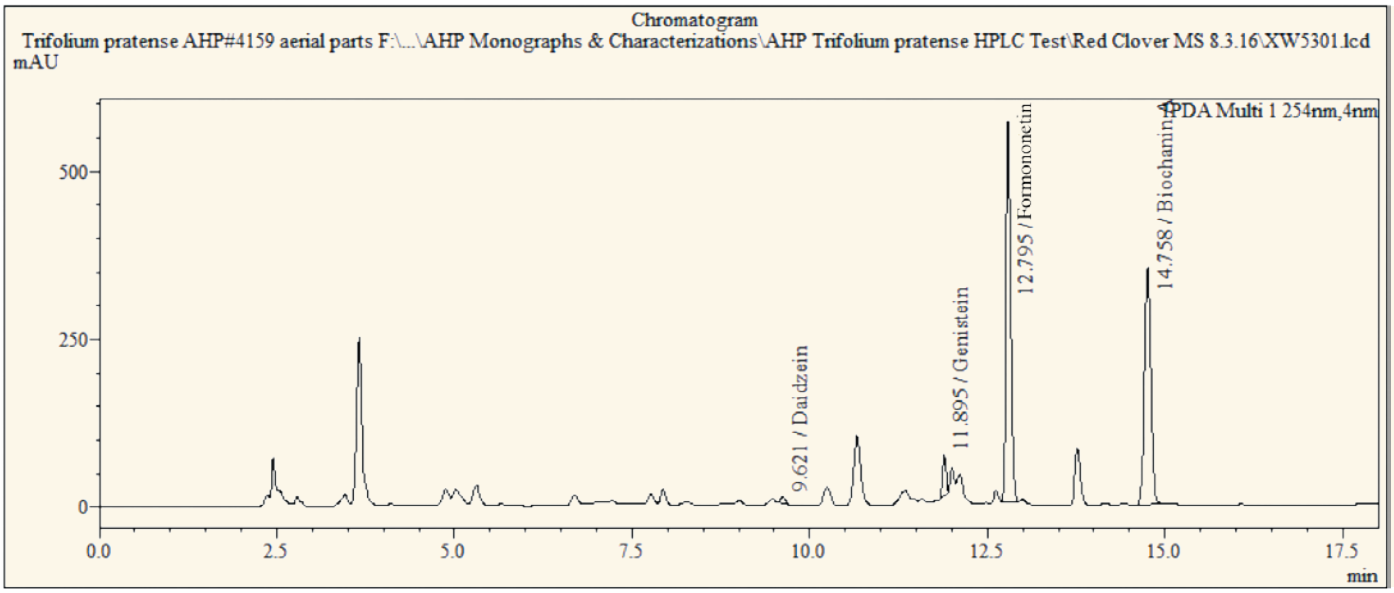


Figure 10a **HPLC chromatogram of red clover aerial parts (leaf, stem, blossom) (with hydrolysis)**

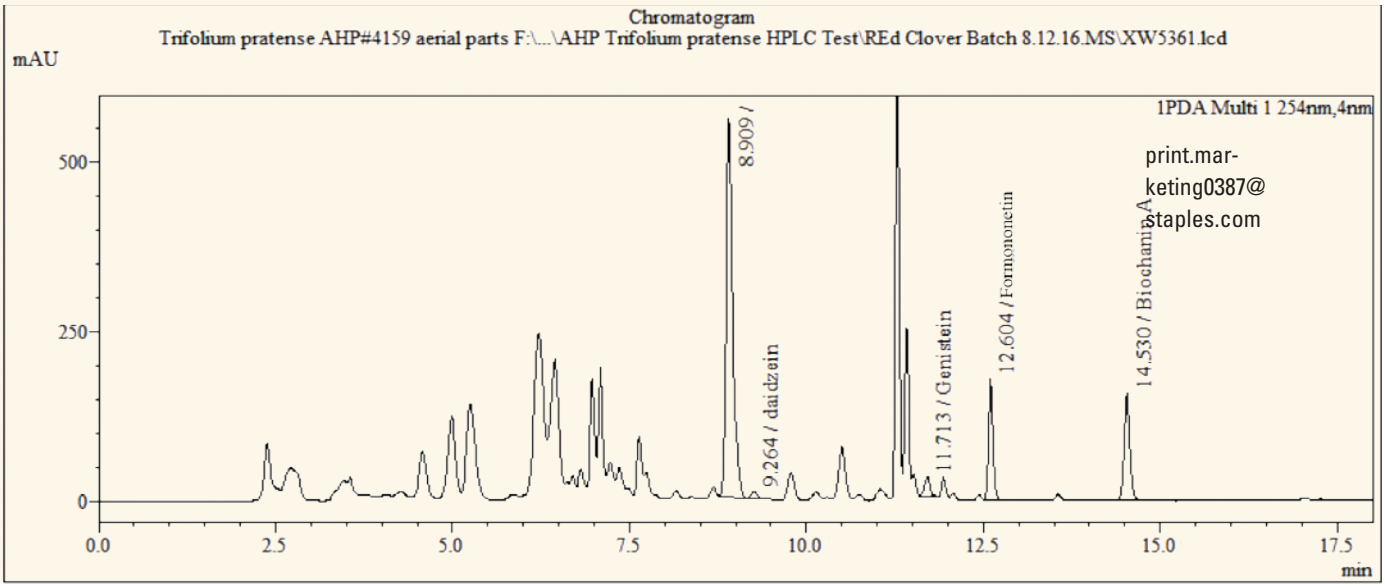


Figure 10b **HPLC chromatogram of red clover aerial parts (leaf, stem, blossom) (without hydrolysis)**

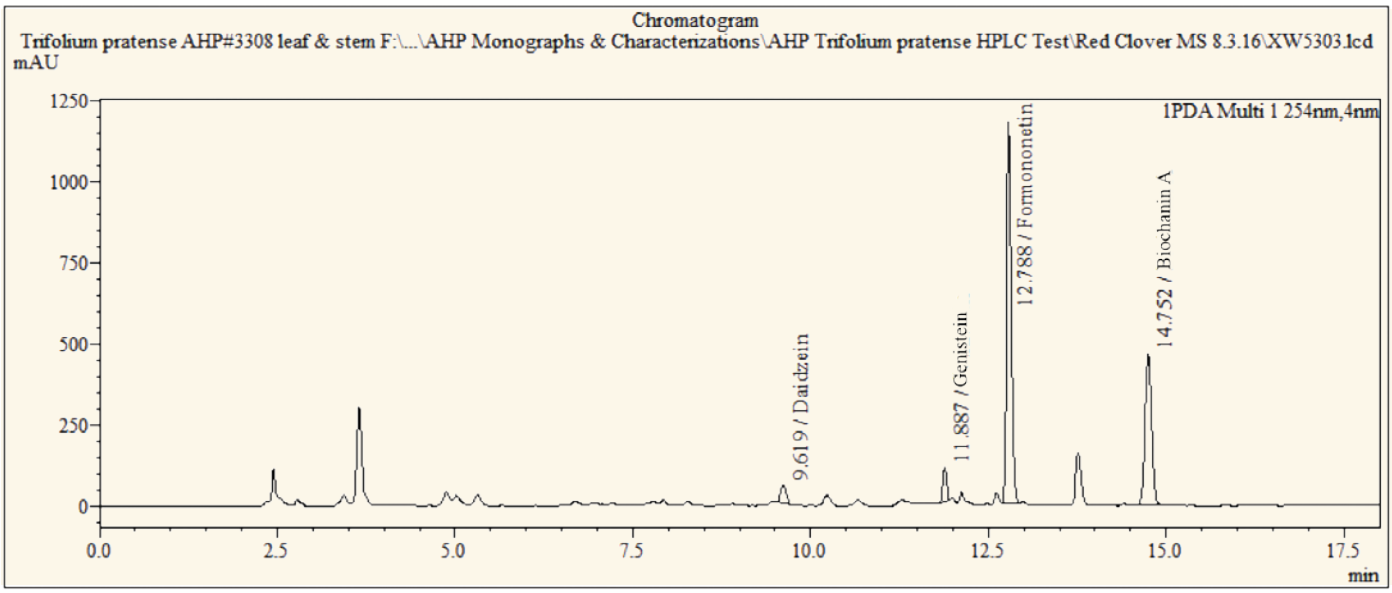


Figure 10c **HPLC chromatogram of red clover leaf and stem (with hydrolysis)**

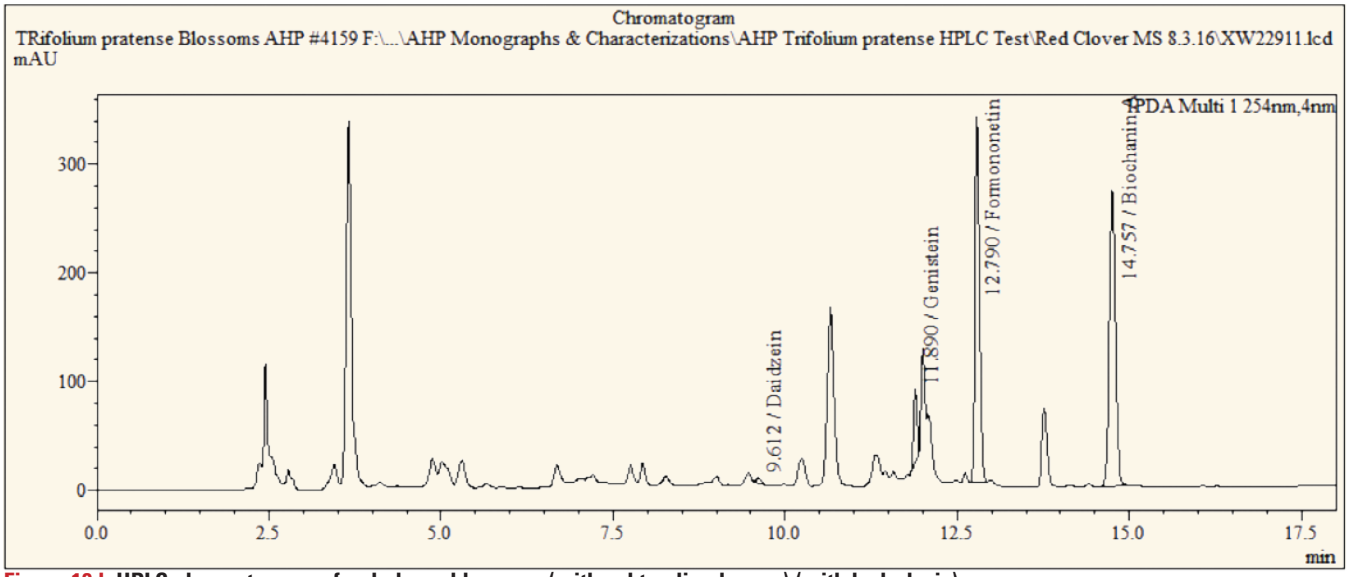


Figure 10d HPLC chromatogram of red clover blossoms (with subtending leaves) (with hydrolysis)

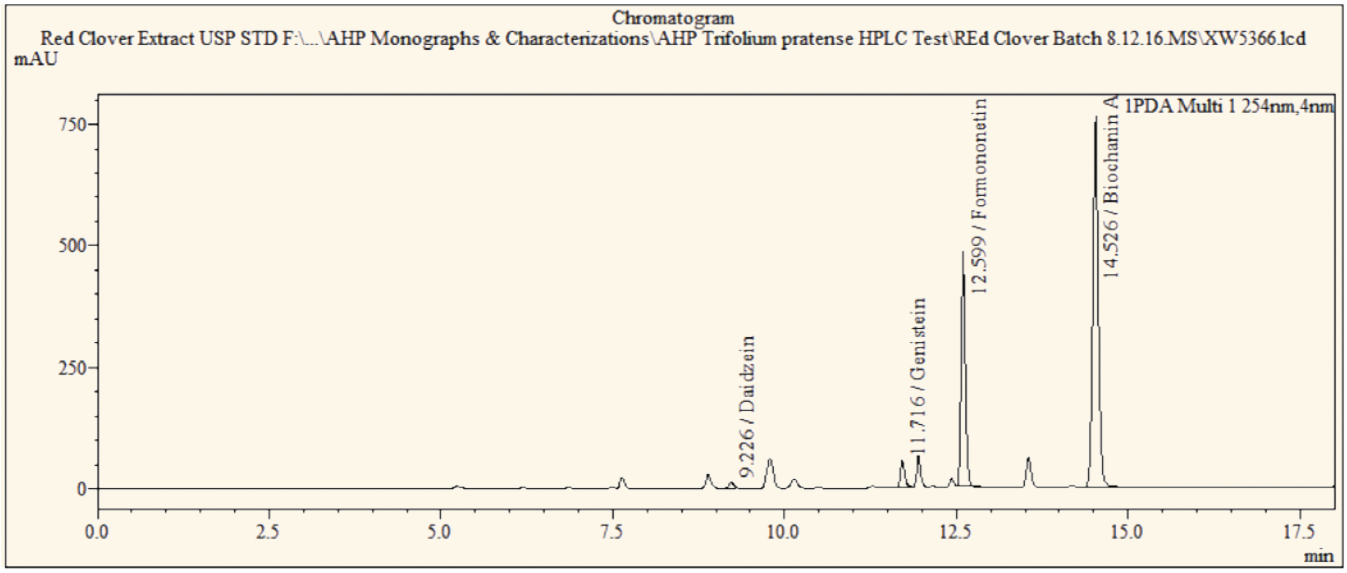


Figure 10e HPLC chromatogram of red clover extract (USP) (without hydrolysis)

Figure 10f HPLC chromatographic comparison of red clover powdered extract (USP) (black) and red clover aerial parts (leaf, stem, blossoms; without hydrolysis) (pink)

Table 4 Pharmacokinetic parameters of biochanin A, formononetin, genistein, and daidzein after long-term administration

	Biochanin	Formononetin	Genistein	Daidzein
Cmin (pre-dose) (ng/mL)	17.3 ± 3.8 (<5.0–43.7)	<5.0 (<5.0–28.6)	54.0 ± 14.1 (<5.0–408.2)	39.9 ± 9.0 (<5.0–116.6)
Cmax (ng/mL)	47.6 ± 5.4 (18.4–79.6)	11.2 ± 2.2 (<5.0–35.4)	114.4 ± 30.0 (42.4–403.6)	62.84 ± 9.4 (28.8–154.2)
Tmax (h)	3.6 ± 0.8 (0.5–11.0)	3.1 ± 0.7 (0.5–9.0)	2.8 ± 0.5 (0.5–6.0)	4.3 ± 0. (0–11.0)
$T_{1/2}$ (h)	17.5 ± 3.0 (4.9–37.7)	22.9 ± 7.8 (4.3–119)	12.9 ± 3. (3.4–39.5)	16.0 ± 3.0 (6.6–54.2)
AUC (ng.h/mL) (0–infinity)	1070 ± 254 (232–3112)	240 ± 96 (40–1424)	2934 ± 836 (382–26,612)	1752 ± 682 (492–10,468)

Source: Howes et al. 2002.

Table 5 Concentrations of urinary isoflavonoids during placebo and isoflavone supplementation

	Placebo		Isoflavone		Difference
Urinary isoflavonoid (mmol/24 h)	Mean ± sem	Range	Mean ± sem	Range	Mean ± sem
Dihydrodaidzein	0.40 ± 0.23	ND–5.0	5.0 ± 1.3*	ND–18.7	4.8 ± 1.2
Dihydrogenistein	0.16 ± 0.16	ND–4.1	4.0 ± 2.3	ND–39.8	3.8 ± 2.1
Daidzein	3.13 ± 0.55	ND–9.3	22.2 ± 2.2*	3.6–45.4	19.1 ± 2.0
Equol	0.16 ± 0.15	ND–3.9	4.3 ± 1.8**	ND–29.6	4.2 ± 1.8
Genistein	0.81 ± 0.26	ND–5.0	7.8 ± 1.5*	0.9–17.5	6.9 ± 0.9
O-dma	0.04 ± 0.04	ND–1.0	9.1 ± 1.5*	ND–24.8	9.0 ± 1.2
Formononetin	ND	ND	13.9 ± 1.5*	2.1–31.1	13.9 ± 1.5
Biochanin A	ND	ND	5.6 ± 0.6*	0.8–10.0	5.6 ± 0.6
Total isoflavones	4.7 ± 1.0	ND–19.3	71.8 ± 5.6*	23.4–127	67.1 ± 5.2

[a] Values are expressed as the mean of four days; n = 25. [b] Difference between placebo and isoflavone supplementations. [c] ND = not detected. Significantly different from placebo: *P <0.0001, **P< 0.03 (paired t-tests). **Source:** Blakesmith et al. 2005.

total isoflavones measured. This experiment suggests that formononetin and biochanin A are readily demethylated to daidzein and genistein. A subsequent study conducted with 14 healthy men and women administered a dose of 80 mg total isoflavones from red clover (Promensil) for two weeks and reported low levels of formononetin and biochanin A in plasma, compared to the metabolites daidzein and genistein (Howes et al. 2002 see Table 4). The AUC for genistein was larger than that for daidzein, approximately 2,934 ng • h/mL and 1,752 ng • h/mL, respectively. The half-life of genistein was 13 hours and that of daidzein was 16 hours.

A study with 12 healthy women administered 50 mg of the glycosides daidzin or genistin and failed to find any traces of the intact glycosides in plasma (Setchell et al. 2002). However the presence of aglycones and conjugates were noted. The authors concluded that daidzin and genistin are not absorbed as glycosides. Absorption takes place following hydrolysis of the sugar moiety to form the aglycone. It was originally thought that the sugars were removed solely by microflora in the colon (Setchell et al. 2002). However, more recent studies have established that deglycosylation is also performed by an enzyme present in the absorptive cells of the intestine, known as lactase phlorizin hydrolase (LPH) (Nielsen and Williamson 2007).

Absorbed aglycones are conveyed from the gut to the liver, where they are conjugated with glucuronate or sulfate before being excreted into the bile to undergo enterohepatic recycling, are released into the systematic circulation, and are finally excreted via the urine (Blakesmith et al. 2005; see Table 5).

An initial study of metabolites in human urine was conducted with seven female participants who took tablets of a red clover dietary supplement (Novogen) which delivered 144 mg isoflavones (Heinonen et al. 2004). Baseline 24-hour urine was collected one day before administration of the supplement. Urine collection was continued for five subsequent days. Using GC-MS analysis of urine samples, it was determined that formononetin is readily demethylated to daidzein and then reduced to dihydrodaidzein, cis-4-OH-equol, *O*-desmethylangolensin, and equol. Biochanin A is

demethylated to genistein and reduced to dihydrogenistein and 6-OH-desmethylangolensin.

Placebo controlled studies conducted with 25 premenopausal women measured levels of isoflavones in urine following supplementation with a red clover isoflavone preparation (Promensil) providing 86 mg/d isoflavones for two to three months (Blakesmith et al. 2005). The urine samples yielded a total of eight different isoflavonoids: formononetin and biochanin A, their demethoxylated analogs daidzein and genistein, the metabolites equol, *O*-desmethylangolensin, dihydrodaidzein, and dihydrogenistein. The mean recovery of individual isoflavones in urine over a 24-hour period was 93% (range 89%–97%). The mean recovery of total isoflavones as a percentage of dose was 22 ± 9% (range 7%–39%). Greater recovery was observed with daidzein and related compounds compared to genistein. Twenty-four hour recoveries in the urine for the daidzein-related structures were: formononetin 13.9 µmol, daidzein 22.3 µmol, *O*-desmethylangolensin 9.1 µmol, and equol 4.3 µmol. Mean recoveries for the genistein-related structures were: genistein 7.8 µmol, biochanin A 5.6 µmol, and dihydrogenistein 4.0 µmol. Recovery of 6-hydroxy-desmethylangolensin was not reported. Biochanin and genistein were measured at 5.6 and 7.8 µmol/24 h, respectively. The effect of dietary protein and fiber on urinary concentrations of isoflavonoids was also explored. The data revealed a significant inverse relationship between urinary isoflavonoid concentration and dietary protein to fiber ratio (r=-0.51, P=0.009), suggesting that isoflavones may adsorb to dietary fiber.

A more recent clinical study with seven adult male and female volunteers who ingested a single dose of a commercial red clover dietary supplement (MenoStabil, Bad Heilbrunner Naturheilmittel GmbH & Co., Germany) reported relatively high concentrations of a metabolite, irilone, in plasma samples (Maul and Kulling 2010). The ingested product contained approximately 38.8 mg total isoflavones calculated as aglycones. Two capsules were administered in a single bolus dose with a glass of water in the morning after fasting overnight and plasma samples were obtained 6.5 hours after ingestion. Analysis of the plasma samples revealed at least nine different isoflavones. The highest plasma concentrations were observed for daidzein (0.385 µM) and irilone (0.351 µM). Formononetin was measured at 0.111 µM, genistein at 0.063 µM, and biochanin A at 0.014 µM. Formononetin and biochanin A were largely demethylated to form daidzein and genistein, respectively. The surprisingly high levels of irilone were speculated to be due to a resistance to degradation by human microflora in the gut (Braune et al. 2010).

Clinical Efficacy and Pharmacodynamics

Phytoestrogens are natural plant-based compounds eliciting a broad array of estrogenic (weak), anti-estrogenic, and estrogen-independent actions. Phytoestrogens include lignans, coumestans, certain flavonoids (e.g., prenylflavonoids) and isoflavones. Specifically, isoflavones occur in plants belonging to the family *Fabaceae*. Interest in the health benefits of phytoestrogens began when correlations were made between isoflavone exposure through soy in the diet, especially the Japanese diet, and lower rates in certain reproductive cancers, cardiovascular disease, and obesity. Increased consumption of phytoestrogens was also linked with a decrease in menopausal symptoms. These reports led to the marketing of supplements containing phytoestrogens, mainly isoflavones, for menopause symptoms. Interest in phytoestrogens increased when the apparent risks of conventional hormonal replacement therapy were revealed.

Much research on this topic has been conducted with soy preparations and soy isoflavones. This monograph summarizes the research conducted with preparations of red clover and provides some data from other isoflavone-containing plants (e.g., soy) that may better inform the understanding of red clover. A randomized, single-blind, placebo-controlled, crossover study comparing 28.5 mg/d soy isoflavone glycosides with 30 mg/d red clover isoflavone aglycone absorption assessed 24-hour urinary excretion of isoflavones following two weeks of daily consumption of each with a two-week washout period (Tsunoda et al. 2002). Approximately 25% of isoflavones were recovered for both sources; 5.82 mg/d daidzein and 1.02 mg/d genistein from soy and 5.26 mg/d daidzein, 1.21 mg/d genistein, 1.02 mg/d formononetin, and biochanin A 0.33 mg/d. Interindividual variability was high, but intraindividual variability was less based on correlations between the amounts secreted from the two sources (r = 0.69; P -0.007).

An important variable to consider regarding studies of isoflavone interventions is the endogenous hormonal status of the subjects in which these substances were administered. This is important because the existing hormonal milieu of the organism may potentially determine how readily exogenous phytoestrogens have access to estrogenic receptors in vivo. For example, in premenopausal women who have higher circulating estrogen levels versus their menopausal counterparts, administration of a high dose of the weakly estrogenic isoflavones might elicit antiestrogenic effects, such as induction of hot flushes, night sweats, etc. In such a context of high endogenous estrogen levels, the isoflavones may compete with the body's natural estradiol for estrogen receptor binding, thus reducing the effect the endogenous (and more potent) estrogens can exert at the receptor binding level. Conversely, in the context of menopause, when endogenous circulating estrogen levels are significantly lower versus in premenopause, high doses of isoflavones may elicit some weak estrogenic effect by binding estrogen receptors in the absence of sufficient estradiol. Whether these effects due to hormonal milieu actually exist needs to be verified.

Finally, the ability of subjects to metabolize isoflavones is an important contributing factor to the observed effect of isoflavones on hormone-related endpoints in clinical studies. Only a portion of the population (25%–50%) may metabolize daidzein into equol, which is known to be a more potent estrogenic metabolite versus other metabolites of the isoflavones. Purified S-equol has recently been shown to have statistically significant effects on clinical endpoints relevant to menopause in both equol-producers and non-

Table 6 Characterization of red clover preparations used in clinical studies

Product, formulation	Individual isoflavones per tablet/capsule (mg) D	G	F	B	Total isoflavones per tablet/capsule (mg)	Studies using preparations
Promensil Novogen, Ltd	0.5	1	16	26	43.5	Atkinson et al. 2004a, 2004b, 2004c; Howes et al. 2000
	5	4	8	25	43	Campbell et al. 2004; Schult et al. 2004
	3.5	4	8	24.5	40	Knight et al. 1999; Nestel et al. 1999
Rimostil	<1	<1	25	2.5	40	Howes et al. 2003, 2004; Schult et al. 2004
P-07 Novogen, Ltd.	3.7	4.3	9.3	25.7	42	Blakesmith et al. 2003
Phytogyn Gynea	NR				NR	Garcia-Martinez et al. 2003
Novogen	1.5	2.5	26	42	40	Heinonen et al. 2004
MF11RCE	NR				40	Imhof et al. 2006
Naturheilmittel GmbH & Co	1.3	0.2	18.9	8.7	38	Maul and Kulling 2010

D = daidzein; G = genistein; F = formononetin; B = biochanin A; NR = no reports of independent chemical analysis found. **Source:** Modified and updated from Booth et al. 2006c.

producers (Utian et al. 2015). This casts uncertainty on previous studies, which did not recruit subjects according to their equol-producing status. The inclusion or lack of assessment of equol producers versus non-producers does not provide clear findings regarding the clinical efficacy of phytoestrogenic substances. Thus, previous studies may have been underpowered to detect a statistically significant effect on estrogenic-related endpoints due to the isoflavone intervention. One report (Utian et al. 2015) summarized studies wherein purified *S*-equol was administered to both equol-producers and non-producers and *S*-equol was found to significantly affect menopausal endpoints in both populations, suggesting that equol is one of the active metabolites that is responsible for reduction of hot flashes, etc. This supports the idea that previous studies that did not select subjects based on their equol-producing status may have been underpowered to detect a significant effect. More studies are needed to verify whether this hypothesis is valid in the context of administering red clover isoflavones. The above findings suggest that previous clinical studies of isoflavone supplements for use in alleviating menopausal symptoms, should be interpreted cautiously and that future studies should pay greater attention to recruitment or assessment of equol versus non-equol producers.

Effects on Menopausal Symptoms

Menopause is characterized by falling levels of estrogen in women along with associated vasomotor symptoms, which include hot flashes (also known as hot flushes), sweating, and sleep disturbances. Isoflavones, found in red clover and soy, have structural similarity with estradiol (17-β-estradiol), and are known to elicit both estrogenic and antiestrogenic effects. For this reason, products made from these plants are popular for use in ameliorating menopausal vasomotor symptoms such as hot flashes and night sweats in women. One should also consider the magnitude of the placebo effect in studies measuring reduction in subjectively measured endpoints, as any such effect may be especially evident in a short-term study (e.g., up to 52%; Freeman et al. 2015). If this phenomenon is present, it may make it more difficult to detect a statistically significant effect of an intervention in a short-term, versus a long-term, study, especially for a mild intervention. Due to funding and other practical concerns, many clinical studies tend to be short-term.

Human Studies

Numerous clinical studies have been conducted evaluating the potential for several different red clover preparations, given at different doses, to reduce menopausal symptoms (see Tables 6–10). Several authors have reviewed these studies in an attempt to come to conclusions regarding safety and efficacy. The individual studies are varied in methodologies and in the red clover products tested. In addition, the reviews themselves have resulted in differing conclusions.

Several clinical studies reported on the incidence of daily hot flushes following treatment with the proprietary red clover product Promensil (Baber et al. 1999a; Jeri 2002; Knight et al. 1999; Tice et al. 2003; van de Weijer and Barentsen 2002). A Cochrane review included a meta-analysis of these studies, which had treatment periods of at least 12 weeks and doses of 40 or 80 mg/d Promensil (Lethaby et al. 2013). Though some of the individual studies (Jeri 2002; van de Weijer and Barentsen 2002) reported significant reductions in hot flash occurrence and frequency, the statistical modeling established for determining efficacy in overall incidence of hot flushes (MD -0.93, 95% CI -1.95 to 0.10, I2 = 31%) in this meta-analysis was not met. Subgroup analysis suggested benefit for a dose of 40 mg/d of Promensil, with

Table 7 Clinical studies of red clover preparations for menopausal hot flashes

Study	Preparation	Isoflavones (mg/d)	Study design	Outcome
Baber et al. 1999b	Promensil	40	DBPCRX; three months + three months; *n* = 51	No change in hot flashes
Knight et al. 1999	Promensil	40	DBPCR; three months; *n* = 37	No change in hot flashes
Nachtigall et al. 1999	Promensil	40	Uncontrolled; two months; *n* = 23	56% reduction in hot flashes
van de Weijer and Barentsen 2002	Promensil	80	DBPCR; three months; *n* = 30	44% reduction in hot flashes
Jeri 2002	Promensil	40	DBPCR; four months; *n* = 30	Frequency: 48.5% reduction vs 11% with placebo (*P* <0.001; Severity: 47% reduction from 2.53 to 1.33 for red clover vs placebo (*P* <0.001)
Tice et al. 2003	Promensil Rimostil	82 57	DBPCR; three months; *n* = 252	No significant change in hot flashes though red clover trended in reducing hot flashes faster than placebo (*P* =0.03)
del Giorno et al. 2010	"Trifolium pratense"	40	RPC; one year: *n* = 100 (50 red clover, 50 placebo group)	No change in hot flashes; placebo performed better than red clover and black cohosh
Geller et al. 2009	Ethanolic extract	120	RDBPC four-arm; one year; *n* = 89)	No change in hot flashes; placebo performed better than red clover and black cohosh
Lipovac et al. 2012	Extract characterized to isoflavones	80	RPCX; 90 days; *n* = 109	Significant reduction of hot flashes and night sweat compared to placebo (*P* =0.0001)
Hidalgo et al. 2005	Menoflavon	80	RDBPCX; 90 days + 90 days; *n* = 60	Significant reduction of hot flashes. Incidence of occurrence in subjects: 15.1% for red clover vs 98.1% for placebo (*P* <0.05); Night sweats: Incidence of occurrence in subjects: 30.2% for red clover vs 92.5% for placebo (*P* <0.05)
Shakeri et al. 2015		80	RPC two weeks; *n* = 72	Highly significant reduction of vegetative and somatic symptoms (*P* =0.0001)

DB = double-blind; PC = placebo-controlled; R = randomized; X = crossover **Source:** Modified and updated from Booth et al. 2006c; Lethaby et al. 2007.

a caveat due to a potential risk of bias. An earlier systematic review and meta-analysis of the same five randomized trials as above came to a different conclusion (Coon et al. 2007). This latter meta-analysis reported a reduction in hot flash frequency in the active treatment group with doses of 40 to 82 mg/d isoflavones compared to placebo (weight mean difference -1.5 hot flashes, 95% CI -2.94 to 0.03; *P* =0.05).

Another, more recent, meta-analysis included the same five aforementioned studies plus three additional studies (del Giorno et al. 2010; Geller et al. 2009; Lipovac et al. 2012) conducted with additional red clover preparations. The results showed that red clover when compared to placebo was effective in reducing menopausal hot flushes when administered for three to four months, by approximately two hot flashes per day (MD = -1.34, 95% CI = -1.90 to -0.77, *P* <0.00001), but this effect did not persist at 12 months (MD = 0.89, 95% CI = -0.07 to 1.85, *P* =0.07) (Gartoulla and Han 2014). All but one of the seven shorter-term studies were conducted with Promensil. Analysis of the two long-term studies, which were conducted with different preparations, indicated no significant effect on menopausal hot flashes. Subgroup analyses based on dose: low (40–80 mg/d, seven

Table 8 Clinical studies of red clover preparations used for bone health

Study	Preparation	Isoflavones (mg/d)	Study design	Outcome
Clifton-Bligh et al. 2001	Rimostil	28.5, 57, or 85.5	DBPCRSB placebo phase; *n* = 46 perimenopausal women; six months	Mid- and high-dose groups had 4.1% and 3.0% increase in BMD of proximal radius and ulna; no change low-dose group
Hale et al. 2001	P-07	50	DBRSB placebo phase; *n* = 46 perimenopausal women; six months	No change in N-telopeptide or osteocalcin bone marker levels
Atkinson et al. 2004a	Promensil	43.5	DBPCR; *n* = 205 (177 after dropout) pre-, peri , and postmenopausal women; one year	Decreased loss of BMC and BMD in lumbar spine in treatment group; no change in hip BMD or bone resorption markers
Schult et al. 2004	Promensil; Rimostil	82 or 57.2	DBPCR; *n* = 252 (245 after dropout) peri- and menopausal women; three months	No change (either treatment) in urinary N-telopeptide or serum osteocalcin levels
Weaver et al. 2009	Rimostil	40	RDBX with two positive controls; *n* = 11; 50 days	No effect on bone resorption
Clifton-Bligh et al. 2015	Rimostil	50	DBPCR; *n* = 142 perimenopausal women; two years	No significant difference in bone density of the spine, femoral neck, or forearm

SB = single-blind; BMD= bone mineral density; DB = double-blind; PC = placebo-controlled; R = randomized; BMC= bone mineral content.
Source: Modified and updated from Booth et al. 2006c.

studies) and high (over 80 mg/d, two studies), did not reveal a dose-related effect. It appears from studies conducted so far that the Promensil product has a marginal benefit in reducing hot flashes and that further studies are needed to reduce the potential risk of bias and improve heterogeneity. Similar findings were reported in a systematic review of Howes et al. (2006).

Summaries of individual studies conducted with Promensil follow. Baber et al. (1999b) reported a study conducted with 51 postmenopausal women that were randomized to placebo and active (one tablet per day of a 40-mg isoflavone supplement) groups in a crossover design trial. There was no significant difference between active and placebo groups in the reduction in hot flushes between start and three months. However there was a strong negative correlation between the level of urinary isoflavone excretion and the incidence of hot flushes. Knight et al. (1999) reported on a 12-week, randomized, double-blind, placebo-controlled, parallel-group trial with 24 postmenopausal women who were randomized to receive a dietary beverage containing isoflavones or an isoflavone-free, isocaloric placebo preparation. No benefit was observed in relief from menopausal symptoms. A parallel placebo controlled sixteen-week study with 30 postmenopausal women given placebo or Promensil reported a reduction in hot flashes compared to placebo (P <0.005) (Jeri 2002). A placebo controlled study that included 30 women reported by van de Weijer and Barentsen (2002) found that treatment with 80 mg/d isoflavones (Promensil) resulted in a significant decrease in hot flushes of 44% between the active and placebo group at 12 weeks, after reaching a maximum reduction of 56% at 10 weeks. Tice et al. (2003) reported on a 12-week randomized, double-blind, placebo-controlled trial in which 252 menopausal women were randomly assigned to Promensil (82 mg/d of total isoflavones), Rimostil (Novogen, Australia; 57 mg/d total isoflavones), or an identical placebo. The reductions in mean daily hot flash count at 12 weeks were similar for all groups. Hot flashes decreased more rapidly in the Promensil group compared to placebo (41%; 95% confidence interval [CI]; 29–51%; P =0.03).

In addition to the studies conducted with Promensil, the Cochrane review included five studies which were conducted with other red clover extracts (Lethaby et al. 2013). One trial, of women (n = 60) using a dose of 80 mg of red clover extract (MF11RCE, Melbrosin Int., Vienna, Austria), for 90 days, reported a significant (P <0.05) benefit for daily frequency of hot flushes and night sweats (Hidalgo et al. 2005). The other two studies, which assessed the efficacy of 40 mg/d red clover (del Giorno et al. 2010) and 120 mg isoflavones/d (Geller et al. 2009), found no difference between groups when treatment was given for 12 months.

A more recent placebo-controlled crossover study, that was not included in the Cochrane systematic review, included 109 postmenopausal women aged 40 or more who were given red clover capsules containing a standardized extract of 80 mg flavone aglycones (Melbrosin Int.) for 90 days in a crossover study design (Lipovac et al. 2012). The endpoints of frequency of daily hot flashes, night sweat and general menopause symptoms measured using the Kupperman Index were all significantly reduced by the red clover extract in comparison to baseline and to the placebo group (P = 0.0001).

Recently, results of a randomized, placebo-controlled clinical trial involving 72 healthy postmenopausal women

Table 9 Subjective quality of life symptoms or condition improvement in postmenopausal women after treatment with red clover extract (RCE†) as assessed with the VAS (crossover trial) over a period of 90 days

Studied parameters	After RCE % improvement	After placebo % improvement	After placebo% improvement	After RCE % improvement
Scalp hair (better texture, less fragility, and overall condition)	7.3 ± 16.6† [0, 0]	4.2 ± 13.9 [0, 0]	0.2 ± 0.9 [0, 0]	6.3 ± 13.9* [0, 0]
Body hair (less growth)	6.4 ± 16.5 [0, 0]	2.6 ± 10.9 [0, 0]	1.0 ± 3.4 [0, 0]	2.2 ± 6.2 [0, 0]
Skin condition (better texture, more moisture, and better overall condition)	18.6 ± 20.5 [15, 32]	6.2 ± 16.2* [0, 0]	5.0 ± 11.0 [0, 5]	17.7 ± 21.2*† [5, 35.0]
Ocular complaint (dryness and burning)	7.8 ± 18.0† [0, 7]	5.3 ± 15.1 [0, 0]	2.2 ± 9.5 [0, 0]	6.0 ± 13.7 [0, 1.3]
Oral mucosa complaint (dryness and burning)	3.9 ± 14.0 [0, 0]	3.3 ± 13.0 [0, 0]	1.8 ± 5.2 [0, 0]	2.8 ± 9.7 [0, 0]
Nasal mucosa complaint (dryness and fissuring)	4.2 ± 15.3 [0, 0]	2.6 ± 9.1 [0, 0]	1.6 ± 5.3 [0, 0]	6.2 ± 16.1* [0, 1.3]
Nails condition (change in thickness and stability)	5.6 ± 16.2 [0, 0]	6.2 ± 16.7 [0, 0]	3.3 ± 10.0 [0, 1.3]	10.8 ± 19.3* [0, 11.3]
Digestive complaints (diarrhea and constipation)	6.0 ± 16.1† [0, 0]	14.4 ± 19.4* [0, 32.0]	19.5 ± 26.0 [6, 31.3]	11.9 ± 26.4* [0, 10.8]
Libido	18.0 ± 16.7† [17, 30]	4.9 ± 14.3* [0, 0]	5.0 ± 12.4 [0, 1.3]	17.8 ± 20.9*† [10, 30]
Urinary complaint (incontinence and dysuria)	5.2 ± 18.1 [0, 0]	5.4 ± 17.3 [0, 0]	3.0 ± 13.7 [0, 0]	4.7 ± 15.0 [0, 0]
Mood complaint	68.5 ± 33.6† [80, 48]	15.0 ± 25.2* [0, 25.3]	7.7 ± 19.2 [0, 0.8]	65.8 ± 37.8*† [78.5, 70.5]
Sleeping complaint	73.5 ± 33.4† [90, 45]	16.2 ± 25.7* [0, 25]	9.8 ± 23.0 [0, 7]	70.6 ± 3.5*† [81.5, 62.2]
Tiredness	61.7 ± 45.8† [96, 100]	16.1 ± 24.5* [0, 26.3]	8.3 ± 22.7 [0, 0]	56.1 ± 47.7*† [92.0, 100]
Overall satisfaction with treatment	87.3 ± 26.6† [100, 12.5]	29.8 ± 31.4* [30, 50]	14.7 ± 26.8 [0, 19]	81.7 ± 26.8*† [100, 43.7]

† RCE: Yielding 80 mg flavone aglycones. Values are expressed as percent improvement and presented as mean ± standard deviations [median, interquartile range]; *P <0.05 when comparing phases in same group using Wilcoxon rank test; †P <0.05 as compared to placebo phase of the contrary group using the Mann-Whitney test. **Source:** Lipovac et al. 2011.

were published by Shakeri et al. (2015). The study revealed a highly significant reduction of severity of menopausal symptoms with 80 mg/d of dried leaves of red clover for 12 weeks (P< 0.0001; 95% confidence interval). Outcome measures were based on the Menopause Rating Scale, the total ending scores in the intervention group decreasing from 20.41 to 10.08 compared to 20.77 to 17.20 in the placebo group.

Effects of Red Clover on Endometrium

The potential for red clover preparations to affect endometrial thickness was assessed in three studies (Baber et al. 1999b; Geller et al. 2009; Imhof et al. 2006). One crossover trial that included 51 postmenopausal women found no difference in endometrial thickness after 12 weeks of treatment with 40 mg isoflavones (Promensil) (Baber et al. 1999b). Another crossover trial that included 109 postmenopausal women reported a significant decrease of 15% in endometrial thickness in women treated with 80 mg of red clover (MF11RCE) for 90 days, compared with no change in women treated with placebo (SD of change not given, P <0.001) (Imhof et al. 2006). The third study, a randomized four-arm, 12-month study that used a noncommercial ethanol red clover extract (398 mg) yielding 120 mg isoflavones/d, found no evidence of a significant difference between groups (Geller et al. 2009). A fourth study, which included 53 postmenopausal women in a randomized crossover design, assessed a dose of 80 mg of red clover extract (Menoflavon) administered for a period of 90 days and reported significant changes in all vaginal cytology indexes (karyopyknotic index, cornification index, maturation index) when compared with placebo (P <0.05) (Hidalgo et al. 2005).

Postmenopausal Depression

A placebo-controlled crossover study that included 109 postmenopausal women aged 40 or more who were given red clover capsules containing a standardized extract of 80 mg flavone aglycones (Melbrosin Int) for 90 days in a crossover study design, assessed depression and anxiety symptoms which were measured using the Hospital Anxiety and Depression Scale (HADS) and Zung's Self Rating Depression Scale (SDS) (Lipovac et al. 2010). Scores of both scales decreased significantly after taking 80 mg flavone aglycones (MF11RCE) for 90 days in comparison to baseline and in comparison to placebo (P <0.001). The reductions in HADS and SDS from baseline with the extract were 76.9% and 80.6%, respectively.

Menopausal Bone Health

Clinical studies that explored the potential for various red clover preparations to have a beneficial effect on bone health in postmenopausal women have yielded variable results. A six-month study of postmenopausal women (n = 46) with doses of 57 or 85.5 mg/d resulted in proximal forearm bone mineral density increases of 4% and 3%, respectively (P =0.002 and 0.023) (Clifton-Bligh et al. 2001). However, in a more robust follow-up investigation by the same researchers, no improvement in bone density was observed. In this double-blind randomized placebo controlled study, 142 women who were menopausal for at least one year were given 50 mg/d isoflavones from a red clover preparation, composed predominately of formononetin and biochanin (P-081, Rimostil), for two years. Both active and placebo groups were also given 1,000 mg/d calcium. Bone mineral density of the spine, femoral neck, and forearm were measured at baseline and at six-month intervals using a densitometer. The data did not show any significant difference between groups in bone density of the spine, femoral neck, or forearm (Clifton-Bligh et al. 2015). These investigators criticized their earlier findings noting the absence of an appropriate placebo group, unexpected large increases in bone mineral density in the forearm, shorter duration time, fewer subjects, and the absence of a dose-response relationship between the fall in the serum LDL cholesterol and increasing amounts of red clover extract. Conversely, supplementation with calcium may have

Another group conducted a study on bone resorption with participants whose bone was prelabeled with ^{41}calcium (Weaver et al. 2009. This was a randomized, crossover blinded study with eleven postmenopausal women that compared four commercial isoflavone preparations (red clover, soy cotyledon, soy germ, and kudzu [*Pueraria* spp.]), two positive controls: estrogen (1 mg) plus progesterone (2.5 mg), and an oral bisphosphonate (risedronate, 5 mg). The intervention periods were 50 days each. Both controls decreased net bone resorption as measured using urinary 41calcium. The red clover product, which delivered 40 mg total isoflavones (Rimostil), had no effect on bone resorption. In contrast, the supplement derived from soy cotyledon, which delivered a total of 220 mg of isoflavones, did reduce calcium loss. This could have been attributed to the low dose of red clover relative to soy and may also demonstrate that different isoflavone doses and/or isoflavone-containing plants are not necessarily equivalent in activity.

A one-year double-blind randomized, placebo controlled study reported a potentially protective effect of red clover on the lumbar spine of women aged 49 to 65 (n = 177) (Atkinson et al. 2004a). The participants received either a tablet of Promensil, providing a dose of 26 mg/d biochanin A, 16 mg formononetin, 1 mg genistein, and 0.5 mg daidzein, or placebo for one year. Compared to placebo, the red clover product significantly reduced the loss of bone mineral content and bone mineral density in the lumbar spine (P =0.04 and 0.03, respectively). There was no significant effect on the hip bone, which is less metabolically active compared to the spine and more difficult to measure.

A randomized, placebo-controlled, double-blind study with 252 menopausal women given either red clover extracts of Promensil (42 mg isoflavones) or Rimostil (28.6 mg/d isoflavones) or a placebo for 12 weeks showed no effects on markers of bone turnover (osteocalcin for formation and urinary N-telopeptide for resorption) at the end of the study (Schult et al. 2004).

In an animal study by Khattab et al. (2014), 12-week treatment with red clover isoflavones (20, 40 or 60 mg/d) resulted in a considerable amelioration of ovariectomy-induced histochemical alterations including improvement of bone structure and decreased level of osteoporosis biomarkers such as procollagen type 1 N-terminal propeptide and osteocalcin.

Cognitive Function in Postmenopausal Women

The potential effect of red clover on cognitive function in postmenopausal women was explored in a randomized placebo-controlled study with 30 postmenopausal women over 60 years of age (Howes et al. 2004). The women were given 56 mg/d isoflavones (Rimostil) for six months. The active treatment improved outcomes of visual-spatial intelligence (isoflavone +12%, placebo -3%, P =0.03), but caused no improvement in verbal memory or digital recall compared to placebo. The authors commented that the findings were not robust and concluded that isoflavone supplementation in this study did not have major short-term effects on cognitive function.

Another study concluded that a red clover supplement had no effect on cognitive function (Maki et al. 2009) based on verbal learning assessment (California Verbal Learning Test: CVLT-modified). This study was a phase II randomized, double-blind, placebo-controlled study with 66 women (mean age 53) who were randomized to receive red clover ethanolic extract (398 mg yielding 120 mg isoflavone glycosides as biochanin A [57.5 mg], formononetin [56.6 mg], genistein [1.6 mg], and daidzein [0.9 mg]), black cohosh ethanolic extract (128 mg), estrogen (0.625 mg) with 2.5 mg/d progesterone supplementation, or matching placebo over a 12-month period.

Quality of Life in Postmenopausal Women

Increasing life expectancy equates to a significant increase

Table 10 Human clinical studies measuring lipid levels in pre- and postmenopausal women

Study	Preparation	Dose (mg/d)	Study Design	Results
Nestel et al. 1999	Promensil	40, 80	RPC, three-stage; 14, 13, 3 (constant placebo) menopausal women, two x five weeks subsequent treatment, eight weeks; run-in/placebo; *n* = 17; 10 weeks	No change in plasma lipids
Baber et al. 1999b	Rimostil	28.5, 57, or 85.5	R, uncontrolled, *n* = 50 postmenopausal women; six months	↑ HDL-C in all groups; ↓ Apolipoprotein B (ApoB)
Knight et al. 1999	Promensil	40/160	DBPCR; *n* = 37 postmenopausal Women; three months	↑ HDL-C in 40 mg group
Nachtigall et al. 1999	Promensil	40	Uncontrolled; *n* = 23 menopausal women; two months	No change in plasma lipids
Howes et al. 2000	Red clover isoflavones (Promensil)	40, 80	RDBX; *n* = 75; postmenopausal women; four weeks	No change in plasma lipids
Clifton-Bligh et al. 2001	Rimostil	28.5, 57, or 85.5	DBR after SB placebo phase. *n* = 46 perimenopausal women; six months	↑ HDL-C↓ ApoB
Hale et al. 2001	P-07	50	DBRPC; *n* = 30 pre-/perimenopausal women; three months	No change in plasma lipids
Garcia-Martinez et al. 2003	Phytogyn	38 mg of red clover isoflavones plus 17 mg of soy isoflavones	Uncontrolled; *n* = 25 postmenopausal women; six months	Significant ↓ TGs
Blakesmith et al. 2003	Red clover isoflavones (Promensil)	86	RDBPC; parallel; *n* = 12 premenopausal women in treatment group; 13 in placebo group; three menstrual cycles	No change in plasma lipids
Howes et al. 2003	Promensil	57	DBPCRX; *n* = 16 postmenopausal type 2 diabetics; four weeks	No change in plasma lipids
Campbell et al. 2004	Red clover isoflavones (Promensil)	86	RDBPCX; *n* = 23; four weeks	↑ HDL-C in postmenopausal women only
Schult et al. 2004	Red clover isoflavones, (Promensil & Rimostil)	82, 57.2	RDBPC, three groups; *n* = 252 (247 after dropout); 12 weeks	↓ TG both (both treatments) for women having ≥å 178 mg/dL at baseline
Nestel et al. 2004	P-07(b) (biochanin A-enriched), P-083 (formononetin-enriched)	40 mg of	DBRPCX; *n* = 46 men and 34 postmenopausal women; six weeks; two parallel groups	P-07(b) ↓LDL by 9.5% in men, no change for women
Atkinson et al. 2004b	Red clover isoflavones, biochanin enriched (Promensil)	43.5	DBPCR; *n* = 205 (177 after dropout) pre-, peri- and postmenopausal women; one year	↓ TGs in perimenopausal women only
Hidalgo et al. 2005	Red clover isoflavones (Menoflavon)	80	RDBPCX; *n* = 60; 13 weeks	↓ TG
Chedraui et al. 2008	Red clover isoflavones (Menoflavon)	80	RDBPCX; *n* = 53; 12 weeks	↓ TC, LDL-C, LpA

Table 10 Human clinical studies measuring lipid levels in pre- and postmenopausal women (continued)

Study	Preparation	Dose (mg/d)	Study Design	Results
Chedraui et al. 2008	Red clover isoflavones (Menoflavon)	80	RDBPCX; *n* = 53; 12 weeks	↓ TC, LDL-C, LpA
Terzic et al. 2009	Red clover isoflavones	40 mg extract: biochanin A (23 mg), daidzein (1 mg), formononetin (15 mg), genistein (1 mg)	PORC; *n* = 22 red clover, 18 not treated; 16 weeks	↓ LDL, TC, TG; ↑HDL-C
Clifton-Bligh et al. 2015	Red clover isoflavones, formononetin enriched (Rimostil)	50	RDBPC; *n* = 97; six months	↓ LDL, TC

R = randomized; DB = double-blind; PC = placebo-controlled; X = crossover; O = open; HDL-C = high-density lipoprotein cholesterol; LDL-C = Low density lipoprotein cholesterol; TC = Total cholesterol; LpA = Lipoprotein A

in the amount of time women spend in a state of estrogen deprivation (menopause) and an increase in the resultant health consequences. In a placebo-controlled crossover trial, menopausal women (n = 109) were randomized to receive either a red clover extract (80 mg standardized to biochanin A, formononetin, genistein, and daidzein) or placebo for 90 days. Subjective assessment using a visual analogue scale (VAS) of skin, appendages, and status of several mucosal sites was carried out for each group. Additionally, other consequences of menopause including libido, tiredness, urinary symptoms, sleep, and mood complaints were also evaluated (Lipovac et al. 2011). A red clover extract yielding a dose of 80 mg/d of biochanin A, formononetin, genistein, and daidzein was given for 90 days with a seven-day washout period prior to crossover. A significant (P =0.05) subjective VAS improvement of scalp hair and skin status, libido, mood, sleep, and tiredness was associated with red clover over placebo. There was no significant difference in urinary complaints, nails, body hair, and mucosa (oral, nasal, and ocular) status. Overall satisfaction with treatment was reported higher after administration of the red clover extract in both assigned groups as compared to placebo.

A more recent placebo-controlled crossover study, that was not included in the Cochrane systematic review, included 109 postmenopausal women aged 40 or more who were given red clover capsules containing a standardized extract of 80 mg flavone aglycones (Melbrosin Int.) for 90 days in a crossover study design (Lipovac et al. 2012). The endpoints of frequency of daily hot flashes, night sweat and general menopause symptoms measured using the Kupperman Index were all significantly reduced by the red clover extract in comparison to baseline and to the placebo group (P =0.0001).

Animal Studies

A rabbit model of menopause (ovariectomy), demonstrated that a 12-week treatment with red clover isoflavones (100 µg/d of daidzein per kg of body weight or a 6.68 mg/kg/d red clover extract estimated as equivalent to 100 µg of daidzein/kg bw) compared to vehicle control, led to significant improvements in bone density, tissue integrity, and vaginal blood flow with minimal effect on uterine weight (Adaikan et al. 2009).

A similar rat model suggested that red clover preparations may improve ovariectomy-induced osteoporosis (Kawakita et al. 2009). The study was conducted using a red clover extract (Menoflavon Forte, Named SpA, Lesmo, Italy) containing 40% of isoflavones (genistein, daidzein, biochanin A, and hydrolyzed aglycones of formononetin). Animals were randomized into four groups: sham-operated rats and three other groups (ovariectomized), being fed for three months as follows: standard food (group B), red clover extract 6 mg/kg/d/animal mixed with food (group C), or red clover extract 6 mg/kg/d/animal with a modified alkaline supplementation through a gastric tube (group D). The ovariectomized rats showed an almost 45% increase of body mass as compared to sham-operated rats but this effect was significantly reduced to less than 30% in both red clover groups. Red clover also ameliorated losses in estradiol levels and uterus weight compared to ovariectomized controls, but these variables were still lower than in sham-operated rats. In addition red clover ameliorated losses in bone mineral density, bone mineral content/bw, and cancellous bone mass in the femoral neck caused by ovariectomy.

In another rat study using the model of ovariectomy-induced osteoporosis, the animals were given red clover isoflavones orally in doses of 20 and 40 mg/bw/animal/d of total isoflavones for 14 weeks (Occhiuto et al. 2007). Treatment with isoflavones significantly increased bone mineral content, mechanical strength of the tibia, femoral weight, femoral density, and prevented the rise of serum alkaline phosphatase levels. In addition, the treatment with isoflavones significantly reduced the number of osteoclasts compared with the ovariectomized control rats. The authors concluded that red clover isoflavones were effective in reducing bone loss induced by ovariectomy, probably by reducing bone turnover via inhibition of bone resorption.

In a study of overiectomized ewes grazing in pastures of either red clover or alfalfa (*Medicago sativa*) high in phytoestrogens, with white clover-ryegrass pasture low in phytoestro-

gens as a control, grazing on red clover proved to be highly estrogenic with large production of cervical mucus and high uterine weights resulting (Kelly et al. 1976). Alfalfa was only mildly estrogenic by comparison. When progesterone was administered by subcutaneous or intramuscular injection, it reduced the mucus production but not the uterine weight response.

In Vitro Studies

Estrogen-Binding Affinity

At least part of the mechanism of action of red clover is the ability of its isoflavones to interact with estrogen receptors (ER). In general, the binding affinity of phytoestrogens is much lower than that of 17 β-estradiol, however their circulating levels in blood can exceed estrogens by 1,000-fold, supplying a rationale for physiological effects (Messina 2010). Isoflavones have a preferential binding affinity to estrogen receptor beta (ER-β) than estrogen receptor alpha (ER-α) and are much more potent at triggering transcriptional activity when bound to ER-β in comparison with ER-α (Messina 2010). Because these receptors have different tissue distributions, and different functions, isoflavone "estrogenicity" and actions will vary markedly from tissue to tissue. The different tissue distributions of ER-α and ER-β and their greater binding affinity and transactivation of ER-β in comparison with ER-α have led to the classification of isoflavones as natural selective estrogen receptor modulators (SERMs) (mixed estrogen agonists/antagonists). SERMs have estrogen-like effects in some tissues but either no effects or antiestrogenic effects in other tissues in which estrogen receptors are present. Research shows that isoflavones affect the expression of many genes differently than estrogen.

In vitro studies using a red clover extract, composed of 35.54% isoflavones (daidzein, genistein, formononetin, biochanin A), 1.11% flavonoids, 0.06% pterocarpans, ≤ 0.03% coumarins, and ≤0.03% tyramine, determined that the extract had an EC_{50} of 2.0–2.2 µg/mL in an alkaline phosphatase (AP) estrogenicity assay, and IC_{50s} of 18.4–32.6 µg/mL and 1.9–3.4 µg/mL in the ERα and ERβ binding assays, respectively (Booth et al. 2006a). Individually, daidzein, genistein, formononetin, and biochanin A were estrogenic in the AP assay, and all of these, except formononetin, bound to one or both ERs.

The binding and transactivating properties of red clover isoflavones was assessed using analysis of estrogen receptors α and β, as well as androgen (AR) and progesterone (PR) receptors (Pfitscher et al. 2008). The affinity of isoflavones to estrogen receptors was as expected, but binding to AR and PR receptors was very weak ($<10^5$ M). Since isoflavones undergo various metabolic transformations after the dietary intake, their metabolites were also analyzed in the binding assays. The study found that the metabolic transformations of isoflavones led to the changes in their affinity to estrogen receptors and in transactivation potential. The demethylation of formononetin and biochanin A to daidzein and genistein, respectively, significantly enhanced binding affinities for estrogen receptor β. The enzymatic conversion of biochanin A, formononetin, daidzein, and genistein to their reduced metabolites dihydrobiochanin A, dihydroformononetin, dihydrogenistein, and dihydrodaidzein, respectively, did not cause any significant effects on the affinity of the compound to either estrogen receptor. Equol, a daidzein metabolite, possessed 30 times the receptor transactivation potential than its precursor daidzein, but its reduced metabolite dihydrodaidzein, displayed approximately 100 times lower transactivation potential than its precursor equol.

Opiate-Binding Affinity

Because the opiate receptor system plays an essential role in the regulation of body temperature, mood, hormone levels, and actions, a red clover extract was investigated for its potential effects on this system. Cell suspensions were incubated with varying concentrations of a proprietary red clover extract (0.3–300 mg/mL; PureWorld Botanicals, Inc.

Table 11 Clinical studies of effects of red clover on breast and endometrial tissue

Study	Preparation	Dose (mg/d)	Study Design	Results
Clifton-Bligh et al. 2001	Promensil	28.5, 57, or 85.5	DBR uncontrolled after SB placebo phase. *n* = 46 perimenopausal women, six months	No change in endometrial thickness
Hale et al. 2001	P-07	50	DBRPC; *n* = 30 pre-/ perimenopausal women; three months	No change in Ki-67 antigen levels or uterine Doppler resistance
Ingram et al. 2002	Promensil	40, 80	DBRPC; *n* = 18; cyclical mastalgia; three menstrual cycles	Significant decrease in breast pain in 40-mg group vs placebo
Atkinson et al. 2004c	Promensil	28.5, 57, or 85.5	DBPCR, uncontrolled, *n* = 177 women at high risk for breast cancer; one year	No change in breast density
Campbell et al. 2004	Promensil	86	RDBPCX; *n* = 23; four weeks	Non-significant decrease in insulin-like growth factor-1 levels in premenopausal women only
Powles et al. 2008	Promensil	28.5, 57, or 85.5	DBPCR pilot study, healthy women (35–70; *n* = 401) at risk for breast cancer; three years	No significant change in breast and endometrium density or FSH

Source: Modified from Booth 2006.

South Hackensack, NJ). The extract displayed a high binding affinity for both μ- and δ-opiate receptors, offering additional pharmacological mechanisms that may contribute to the putative effects of red clover for reducing menopausal symptoms (Nissan et al. 2007).

Metabolic Syndrome/Cardiovascular Health

Human Studies

Metabolic syndrome is defined as a cluster of three of the following four risk factors: elevated blood glucose levels, elevated blood pressure, abnormal lipid profile (elevated cholesterol levels), and excess body fat around the waist. Estrogen therapy is reported to improve insulin sensitivity, improve arterial elasticity thereby improving blood pressure, and promote a favorable lipid profile in peri- and postmenopausal women. However estrogen therapy is associated with a slight increase in the risk of strokes, blood clots, and other problems. When combined with the hormone progestin, the risks of breast cancer and heart attack may rise as well (Anderson et al. 2006; Billeci et al. 2016; Chlebowski et al. 2010)

As red clover extracts have been investigated as a potential alternative for hormone replacement therapy, they have been investigated for their role for reducing cardiovascular disease risk factors, the results of which have been mostly positive. The primary functions of phytoestrogens in metabolic syndrome have been summarized as follows (Jungbauer and Medjakovic 2014). Phytoestrogens are involved in the down-regulation of pro-inflammatory cytokines, such as COX-2 and iNOS; increase PPAR-mediated reverse cholesterol transport and PPAR-medicated insulin sensitivity; possess antioxidant activity; and effect adipogenesis.

Effect on Lipid Levels

At least 18 clinical studies examined lipid levels in peri- or post-menopausal women following intake of red clover preparations, with one study including men (see Table 10). A number of these reported positive findings, while a number did not. Two studies reported significant reductions in triglycerides following intake of approximately 60 to 80 mg/d of isoflavones (Promensil, Rimostil, Menoflavon) for 12 or 13 weeks (Hidalgo et al. 2005; Schult et al. 2004). A study with postmenopausal women using 40 mg/d red clover isoflavones for 12 months reported a significant decrease in serum triglycerides, total cholesterol and low-density lipoprotein (LDL)-cholesterol, as well as a significant increase in high-density lipoprotein (HDL)-cholesterol (Terzic et al. 2009). Another study similarly reported significant reductions of serum LDL and total cholesterol following intake of 50 mg red clover isoflavones (Rimostil) for one year (Clifton-Bligh et al. 2015). Still another study of postmenopausal women significantly reduced serum LDL and total cholesterol along with lipoprotein A levels, this time with 80 mg red clover isoflavones for 90 days (Chedraui et al. 2008). One study reported significant reductions of serum LDL and total cholesterol following intake of 50 mg red clover isoflavones (Rimostil) for one year (Clifton-Bligh et al. 2015). Another study found a dose of 86 mg isoflavones (Promensil) increased HDL in postmenopausal women compared to placebo; however there were only seven women in this group and there was no change in the 16 premenopausal women (Campbell et al. 2004). This is consistent with the proposal that the hormonal milieu affects the relative effects isoflavones may exert at estrogenic receptors in vivo.

Several studies reported no changes in serum lipids in menopausal, perimenopausal, and postmenopausal women compared to placebo controls with doses of red clover isoflavones of 40 or 80 mg (Promensil) for four weeks, 10 weeks, three menstrual cycles, or one year (Atkinson et al. 2004b; Blakesmith et al. 2003; Howes et al. 2000, 2003; Nachtigall et al. 1999; Nestel et al. 1999). Another study examined a product that combined 60.8 mg of red clover isoflavones plus 19.2 mg soy isoflavones compared to placebo in healthy postmenopausal women (67 in the active group; 61 in the placebo group). No significant effect on lipids was observed after six months (Mainini et al. 2013).

A randomized, placebo-controlled, double-blind crossover study with 46 men and 34 women, with an average age of 58 years and comparable plasma LDL-cholesterol levels, compared treatments with 40 mg/d of a red clover extract enriched in either formononetin or biochanin A or placebo for six weeks each (Nestel et al. 2004). Men receiving biochanin (genistein precursor) treatment had a significantly lowered LDL-cholesterol by 9.5% compared to placebo, whereas women and those receiving formononetin (daidzein precursor) treatments did not.

Animal Studies

Pure red clover was added to a standard rabbit chow diet (8%) and fed to hyperlipidemic rabbits. Red clover supplementation resulted in a significant decrease in C-reactive protein (CRP), triglyceride (TG), total cholesterol, and LDL-cholesterol (LDL-C) whereas, HDL-cholesterol (HDL-C) was significantly increased ($P < 0.05$). There was also evidence of a diminishment of aortic and arterial fatty streak formation providing some mechanistic support of the potential of red clover to reduce cardiovascular and atherosclerotic risk (Asgary et al. 2007).

Another study looked at the potential of a red clover extract (Rimian, Fuzhou, China; standardized to 10% isoflavones (consisting of 5.1% formononetin, 4.8% biochanin A, 0.16% genistein, and 0.04% daidzein) to affect dietary-induced non-alcoholic steatohepatitis (fatty liver disease) (Chen et al. 2014b). Extracts were administered orally at 50 and 200 mg/kg/d for 35 days. While hepatic steatosis (fatty infiltration of liver cells) was significantly reduced ($P < 0.05$), inflammation was not.

Insulin Sensitivity

In a three-month randomized study, 43 healthy (non-diabetic) postmenopausal women (mean BMI 27 mg/m^2) were divided into four groups: 0.05 or 0.1 mg transdermal estrogen therapy compared with 40 or 80 mg/d oral Promensil (Lee et al. 2012). After three months, insulin sensitivity was reduced in the red clover groups compared to the estrogen

groups. There were no significant changes in either group for fasting glucose or insulin levels once adjustments were made for differences in age and BMI. Transdermal estrogen therapy significantly increased sex hormone-binding therapy (SHBG), but there were no changes in SHBG in the red clover groups.

A randomized, double-blind, placebo-controlled, parallel study with 25 healthy premenopausal women found no significant effect on glucose or insulin concentrations with 86 mg/d of red clover isoflavones in the treatment group of 12 subjects (Blakesmith et al. 2003).

Blood Pressure

A randomized, placebo-controlled study examined the effects of supplemental red clover isoflavones on blood pressure (Howes et al. 2003). Sixteen postmenopausal women with type 2 diabetes treated with oral hypoglycemic therapy or diet completed a four-week crossover study in which they received approximately 55 mg/d isoflavones (50 mg formononetin, 5.0 mg biochanin, and less than 1 mg genistein and daidzein; Novogen Pty Ltd, Australia) or placebo. Twenty-four hour ambulatory blood pressure recordings and forearm vascular responses to the vasoconstrictors acetylcholine, nitroprusside, and L-nitromonomethylarginine were measured at the end of each treatment period. Compared to placebo, the isoflavone preparation caused a significant mean reduction in daytime blood pressure (systolic and diastolic, -8.0 ± 3.4 and -4.3 ± 1.9 mmHg, respectively, $P < 0.05$). There was no significant effect on nighttime blood pressures. Isoflavone supplementation significantly increased the response to the nitric oxide synthase inhibitor L-nitromonomethylarginine (L-NMMA), but not to acetylcholine. The authors of the study suggested that the isoflavones improved basal, but not stimulated, forearm endothelial function.

In another study, arterial compliance was increased by an average of 23% in 17 menopausal women receiving 80 mg of red clover isoflavones for five weeks, in comparison with placebo (Nestel et al. 1999). The effect of a 40 mg isoflavone dose was approximately 20%.

In Vitro Studies Metabolic Syndrome

Red clover extracts are reported to affect functions of peroxisome proliferator-activated receptor (PPAR)δ, an important regulator of adipocyte differentiation, fatty acid storage, glucose metabolism, and insulin sensitization. The genes activated by PPAR δ stimulate lipid uptake and adipogenesis by fat cells. Researchers reported that red clover extracts and the compounds genistein and biochanin A were potent PPARδ ligands and activators (Mueller and Jungbauer 2008; Mueller et al. 2010). Several isoflavone metabolites exerted greater binding affinities or transactivational activities than their precursor molecules. For example, 6-hydroxydaidzein had a more than 100-fold higher binding affinity in comparison to its precursor daidzein. The observed maximal transactivational activity of 6-hydroxy daidzein and 30-hydroxygenistein exceeded the action of rosiglitazone, a PPAR δ agonist (Mueller and Jungbauer 2008).

Effects on Cancer

Human Studies

Effects on Breast Cancer

Endogenous and supplemental estradiol have a tumorigenic effect on some types of cancers. Because of this, there is concern that phytoestrogens may have a similar tumorigenic effect. A number of studies suggest that phytoestrogens, while tumorigenic in some cancer cell lines in vitro, are not tumorigenic in vivo. Numerous studies provide mechanistic rational of how phytoestrogens differ from endogenous estrogens in this regard and, in vivo, elicit an anti-tumorigenic effect (see Safety and Discussion on Phytoestrogens). Some phytoestrogen exposure studies report modest protective effects of phytoestrogens; others detect no association between phytoestrogen intake and breast cancer risk; and a few have reported marked protective effects of phytoestrogens against breast cancer (Hirohata et al. 1985; Hirose et al. 1995; Key et al. 1999; Nomura et al. 1978, 1985; Trock et al. 2006; Wu et al. 1996; Yuan et al. 1995).

A meta-analysis on randomized, controlled clinical trials monitoring the effect of isoflavones on mammographic breast density, a biomarker of breast cancer risk, identified eight studies with a total of 1287 women that met inclusion criteria (Hooper et al 2010), two of which utilized a red clover isoflavone extract (Promensil) (Atkinson et al. 2004c; Powles et al. 2008). The meta-analysis indicated no overall effect on isoflavones on breast density for all women combined or for postmenopausal women, though there was a modest increase in premenopausal women. However, when using the Powles (2008) study *P*-value data rather than confidence interval (CI) data, the sensitivity analysis resulted in a marginal loss of statistical significance for the premenopausal subgroup (Hooper et al 2010). The Atkinson (2004c) study of 177 women total (16% premenopausal, 14% perimenopausal, 67% postmenopausal) indicated that 43.5 mg/d red clover isoflavones for one year failed to increase mammographic breast density.

Like high levels of estradiol, high levels of insulin-like growth factor-1 are associated with an increased risk of premenopausal breast cancer. A randomized, placebo-controlled, double-blind crossover study that included 16 premenopausal women and seven postmenopausal women who received 86 mg/d red clover isoflavones (Promensil) or placebo for one month, with a minimum two-month crossover period, measured levels of insulin-like growth factor in plasma (Campbell et al. 2004). This study showed no significant changes in the levels of insulin-like growth factor-1 with supplemental isoflavones compared to placebo.

Animal and In Vitro Studies

Researchers studied if red clover-derived isoflavones (Menoflavon) exert direct actions in breast cancer cells regulating invasion and migration of the cells (Mannella et al. 2012). A mix of compounds found in red clover, including genistein alone (concentrations not given), slightly increased T47- D human breast cancer cell migration and invasion. However, in the presence of estradiol, the isoflavones acted

as antiestrogens, blocking both horizontal cell migration and invasion of three-dimensional matrices induced by estradiol. The actions on breast cancer cell motility appear to be through regulation of the actin cytoskeleton of breast cancer cells via a set of molecular steps that converge on the actin-binding protein moesin.

Other studies showed that formononetin inhibited breast cancer cell migration and invasion in a dose-dependent fashion by reducing the expression of matrix metalloproteinase (MMP)-2 and MMP-9 through the PI3K/AKT signaling pathway. The same researchers reported that formononetin inhibited breast cancer metastasis and prolonged animal survival time in vivo (Zhou et al. 2014).

In other receptor binding assays, Spagnuolo et al. (2014) reported that red clover and isoflavone extracts (Promensil) stimulated estrogen-sensitive breast cancer cell lines (MCF-7) in descending order of potency: genistein, biochanin A, daidzein, formononetin. In four of five extracts tested, the strongest binding affinity (mean EC_{50}) was correlated more with lower ratios of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavone (lower content of biochanin A and higher content of formononetin) than with inverse ratios of these or higher concentration of individual isoflavones. Red clover isoflavones were active in the nM–µM range at a level that was not significantly different than estradiol (E2). This study suggests the MCF-7 binding assay may be an appropriate biological assay that can be coupled with quality control and product standardization. The authors also noted that factors other than isoflavone concentration contributed to the activity of the entire extract in that the red clover extract differed in effect than fixed combinations of individual isoflavones, suggesting that undefined co-factors in the extract are playing a role (Spagnuolo et al. 2014). Antiproliferative effects have been demonstrated for other compounds in red clover such as O-desmethylanglolensin, which significantly inhibited MCF-7 cell proliferation after 48 and 72 h in a dose- and time-dependent manner (306.34 and 178.52 µM at IC_{50} for 48 and 72 h, respectively ($P < 0.05$). Cell proliferation was decreased by 55.15% compared with controls after treatment with 200 µM *O*-desmethylanglolensin for 72 h (Choi and Kim 2013).

Effects on Prostate Cancer

Human Studies

The potential benefits of a red clover preparation for men diagnosed with prostate cancer were examined in a study using archival tissue from matched controls. Twenty men who were diagnosed with non-metastatic prostate cancer and had a Gleason score of ≥5 based on pathological assessment from biopsy specimens were matched with men of the same age with similar prognostic markers of prostate cancer (Gleason score, preoperative prostate specific antigen [PSA] levels and metastatic markers [TNM] stage) (Jarred et al. 2002). The men in the treatment group received four tablets/d (Trinovin; Novogen, Sydney, Australia) containing 160 mg/d of red clover-derived dietary isoflavones (a mixture of genistein, daidzein, formononetin, and biochanin A) for the period of time available before their scheduled radical prostatectomy (median 20 days, range seven to 54 days). There were no significant differences between pre- and post-treatment serum PSA, Gleason score, serum testosterone, or biochemical factors in the treated patients ($P > 0.05$). The incidence of apoptosis in prostate tumor cells from radical prostatectomy specimens was compared between 18 treated and 18 untreated control tissues. Apoptosis in radical prostatectomy specimens from treated patients was significantly higher than in control subjects ($P = 0.0018$), specifically in regions of low to moderate-grade cancer (Gleason grade 1–3). The authors of the study speculated that isoflavones might halt the progression of low to moderate-grade prostate cancer by inducing apoptosis in cancer cells.

A case-controlled study examined a possible correlation between plasma concentrations of phytoestrogens and risk of subsequent prostate cancer in 950 men with incident prostate cancer and 1,042 control subjects, participating in the European Prospective Investigation into Cancer and Nutrition (EPIC) (Travis et al. 2009). There were no statistically significant associations for circulating concentrations of daidzein, equol, enterolactone, or enterodiol in relation to overall risk for prostate cancer. In contrast, initial analysis indicated that higher plasma concentrations of genistein were associated with lower risk of prostate cancer. However, when the analysis was repeated with an increased number of participants (1,605 prostate cancer cases and 1,697 matched controls) the analysis revealed that plasma genistein concentrations were not associated with prostate cancer risk (Travis et al. 2012).

A 60 mg/d dose of a standardized water and ethanol isoflavone extract of red clover (with genistein, daidzein, formononetin, and biochanin A) was taken for one year by 20 men of mean age of 60 years with elevated prostate-specific antigen (PSA) and negative prostate biopsy findings (Engelhardt and Riedl 2008). The average PSA level of 10.16 ng/mL at baseline was significantly reduced to 7.15 ng/mL after 12 months ($P < 0.019$). The average prostate volume was reduced from 49.3 cm^3 to 44.3 cm^3 ($P < 0.097$). Sexual hormone levels and sexual function were not influenced.

According to a case report, a 66-year-old man with high-grade prostate adenocarcinoma took four 40 mg/d Promensil tablets for one week prior to a prostatectomy. The resected prostatic specimen showed prominent apoptosis indicative of tumor regression, an effect typical of a response to high doses of estrogen (Stephens 1997). No adverse side effects occurred.

In Vitro Studies—Prostate Cancer

Red clover isoflavone treatment led to a dose-dependent decrease in prostate-specific antigen (PSA) protein and gene expression and testosterone metabolism induced by transforming growth factor (TGF)–β1 plus dehydro-epiandrosterone (DHEA) in an in vitro prostate tissue model where stromal (6S) cells and epithelial (LAPC-4) cells were co-cultured (Gray et al. 2009). The isoflavones biochanin A, formononetin, daidzein, and genistein (Sigma) were dissolved in DMSO and combined in the same proportions as the

published formulation in the clinically used Promensil and tested in a concentration range of 10 nM and 300 nM. In further studies, the mechanisms controlling those activities were explored (Liu et al. 2011). They suggested a pathway connecting overproduction of TGFβ with increased PSA in prostate cancer and partial suppression by isoflavones.

Formononetin demonstrated an inhibitory effect on the proliferation of both LNCaP and PC-3 prostate cancer cell lines (Ye et al. 2012). The molecular mechanisms of action involve inhibition of extracellular signal-regulated kinase1/2 (ERK1/2) mitogen-activated protein kinase (MAPK) signaling pathway, leading to the increased expression of BCL2- associated X (Bax) mRNA and protein, and induced apoptosis. Another research group presented evidence that formononetin triggers cell apoptosis in human prostate cancer cell DU-145 through the mitochondrial apoptotic pathway by up-regulating RASD1 (dexamethasone-induced Ras-related protein 1) (Liu et al. 2014).

Colon Cancer

Human Studies

A randomized, double-blind, placebo-controlled crossover study for eight weeks investigated the effect of red clover isoflavone supplementation (84 mg/d) on serum insulin-like growth factor (IGF) concentrations, which are linked to risk of colorectal cancer (Vrieling et al. 2008). The isoflavone preparation (Promensil) contained 42 mg of total isoflavones (25 mg biochanin, 8 mg formononetin, 4 mg genistein, and 5 mg daidzein). The study, which included 34 postmenopausal women with a family history of colorectal cancer or personal history of colorectal adenomas, indicated that the isoflavones did not significantly affect circulating levels of IGF. Colorectal tissue obtained via biopsy, indicated no change in mRNA expression of IGF system components. These data suggest that isoflavones do not significantly affect circulating levels of IGF and thus do not have an influence on the risk of colorectal cancer as associated with this marker.

Animal and In Vitro Studies

Anti-Angiogenic and Anti Proliferative Potential

Investigations suggest that phytoestrogens may inhibit angiogenesis and thereby act as chemo- preventive agents. Evaluation of the anti-angiogenic action of red clover extracts (8.5% isoflavones; Melbrosin Int., Vienna, Austria), with the use of the chorioallantoic membrane assay in fertilized hen's eggs, demonstrated a considerable inhibition of angiogenesis. The effect due to a dosage of 250 mg of extract per pellet, one pellet per egg, was rated as a "good" anti-angiogenic effect. Non-methylated isoflavones: daidzein and genistein (50 µg/pellet) were found to possess stronger anti-angiogenic activity (very good and good, respectively) than the methylated compounds: formononetin and biochanin A (none and weak, respectively) (Krenn and Paper 2009).

A study investigated whether isoflavones might have an anti-proliferative effect similar to estrogen in benign prostate hyperplasia (Slater et al. 2002). Male Wistar rats were fed a diet that contained 5% red clover extract (Novogen Ltd), control diets containing no isoflavones, or standard rat pellets (not characterized) for 14 months. At that time, the animals were euthanized and their prostates examined using immunohistochemistry. Red clover isoflavones in the diet produced a significant increase in the production of estrogen receptor β and the adhesion protein E-cadherin but a decrease in the label for transforming growth factor β1. These proteins are estrogenically modulated markers for proliferation, maintenance of histological architecture, preservation of cell phenotype, and reduction of the potential for neoplastic and metastatic transformation. The authors indicated that the study suggested that red clover might be beneficial in preventing neoplastic transformation leading to cancer.

Angiogenesis

Isoflavones, including genistein and daidzein, down regulate genes and mRNA levels of proteins involved in angiogenesis such as IL-8, matrix metalloproteinase 13, and fibronectin (Kolodziejczyk-Czepas 2012). Moreover, the synthesis of anti-angiogenic factors: plasminogen activator inhibitor-1 (PAI-1), angiostatin, and thrombospondin appear to be up regulated by isoflavones.

Antioxidant Activity

Human Studies

The overproduction of reactive oxygen and nitrogen species (ROS/RNS) is involved in the etiology and pathophysiology of inflammation and cancer, as well as autoimmune, neurodegenerative, cardiovascular and other disorders. Antioxidant activities for red clover preparations have been demonstrated in vitro, but not as yet in human clinical studies.

A randomized, placebo-controlled, double-blind crossover study that included 16 premenopausal women and seven postmenopausal women who received 86 mg red clover isoflavones (Promensil) or placebo for one month, with a minimum two-month crossover period tested levels of antioxidants in serum (Campbell et al. 2004). There was no effect from the isoflavones compared to placebo on levels of vitamin E, vitamin C, or malondialdehyde in fasting blood samples in these women.

Animal and In Vitro Studies

A study with mice revealed antioxidant actions of red clover-derived formononetin (0.05 g/kg bw or 0.5 g/kg/d bw, administered orally for six months to ovariectomized animals (Mu et al. 2009). The intake of formononetin significantly increased the activities of superoxide dismutase (SOD), glutathione peroxidase, catalase, and reduced lipid peroxidation. A methanol extract of red clover demonstrated the ability to reduce 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging (EC50 value 112 µg/mL), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging (EC50 94 µg/mL), superoxide anion radical scavenging (75 µg/mL) and hydrogen peroxide scavenging

activity (103 µg/mL) (Khorasani et al. 2015). A significant correlation was found between antioxidant activity and the presence of phenolic and flavonoid constituents. In addition, a methanol extract of red clover and a phenolic fraction in final concentrations of 1.5–50 µg/mL partly protected human blood plasma against the action of 200 µM peroxynitrite in vitro (Kolodziejczyk-Czepas et al. 2015). A phenolic fraction of red clover displayed stronger free radical scavenging properties estimated by the reduction of ABTS and DPPH radicals (EC_{50} values 22 and 12 µg/mL, respectively) compared to the crude extract. A previous study by this group demonstrated a red clover extract (0.5 to 50 µg/mL) provided a protective action for human blood platelets exposed to oxidative stress from 100 µM peroxynitrite in vitro (Kolodziejczyk-Czepas et al. 2013, 2015). The extract considerably reduced the peroxynitrite-mediated modifications of proteins and diminished peroxidation of lipids in platelets. Other studies have similarly reported antioxidant activity of red clover flower extracts characterized on flavonoids with IC_{50} values of 34.0 µg/mL. White clover (*Trifolium repens*) showed stronger antioxidant activity with IC_{50} values of 21.4 and 10.3 in ABTS and DPPH assays, respectively and corresponded with flavonoid concentrations of quercetin, rutin, and chlorogenic acid (Tundis et al. 2015).

Antioxidant activity has also been demonstrated for the essential oil of red clover, with radical-capturing capacity for DPPH (IC_{50} value 27.61 µg/mL), NO (16.03 µg/mL), O2 (16.62 µg/mL), as well as reducing lipid peroxidation effects in the Fe2+/ascorbate induction system (9.35 µg/mL) (Vlaisavljevic et al. 2014). Another study reported on the antioxidant activity of phenolics and flavonoids (Esmaeili et al. 2015). In this study, methanol extraction yielded the highest concentration of phenolics and flavonoids and was correlated with the greatest antioxidant activity.

A number of other studies have articulated additional actions that may contribute to a cancer protective effect of red clover and its isoflavones. Biochanin A derived from red clover was shown to inhibit benzopyrene-induced carcinogenesis in cell cultures with varying fractions resulting in varied levels of inhibition (22%–30% for water-soluble fractions; 30%–41% for ethyl alcohol fraction) as compared to a DMSO control (Cassady et al. 1998). In another study, biochanin A, daidzein, formononetin, and genistein significantly ($P < 0.001$–$P < 0.05$) inhibited the inflammatory mediator cyclooxygenase (COX2) in a murine macrophage cell line (RAW 264.7) at 1–40 µM and in human monocytes at 10-100 µM (Lam et al. 2004).

Wound Healing Effects

Red clover leaves and blossoms are used topically as an anti-inflammatory and for promoting wound healing in Turkish folk medicine and various species of *Trifolium* were investigated for this putative effect. Using linear and circular incision wound models, an ointment (1% concentration) prepared from an aqueous methanolic extract of *T. pratense* and *T. canescens* was applied to wounds and compared against a control (Madecassol®, prepared from *Centella asiatica*). Increased wound tensile strength and tissue contraction for both *T. canescens* (356% and 48.96%, respectively) and *T. pratense* (21.3% and 30.21% respectively), were reported. The authors suggest that isoflavone content was correlated with activity but provide no real data to demonstrate this (Renda et al. 2013).

In a study by Cecotti et al. (2013), significant antibacterial activity of the volatile compounds of red clover oil (yielding 0.006% to 0.11%) was demonstrated against *Paenibacillus*, *Melissococcus plutonius*, and *Bacillus subtilis* ($P < 0.05$). The first two bacterium cause foul brood in bees, the latter, at one time was cultured as a probiotic and used as an immunostimulatory agent for the treatment of gastrointestinal and urinary tract disease (Ciprandi et al. 1986). These antibacterial actions may partly contribute to the putative wound-healing activity attributed to red clover. The antibacterial action of the oil (2 mg/mL) was not as great as that of an equivalent concentration of oxytetracycline hydrochloride. The use of this probiotic declined with the advent of antibiotics.

Conclusion

There is no data on the use of red clover for any of its historical uses as an alterative (see Traditional Western Herbal Medicine Supplement), blood purifier, or antitussive.

Clinical findings based on current studies suggest that red clover extracts are of moderate benefit in reducing hot flashes, may reduce postmenopausal depression, anxiety, and spinal bone loss, and improve some postmenopausal quality of life parameters (scalp hair, skin, mood, sleep). Due to growing understanding of the role the daidzein intestinal metabolite equol plays in determining clinical endpoints, especially in menopause, and that not all subjects produce equol equally, previous studies may have been underpowered in determining efficacy. Future studies should also look at the hormonal status of subjects to determine the optimal application of phytoestrogens in general and red clover specifically.

In vitro studies demonstrate that, red clover isoflavones and isoflavone-containing red clover extracts interact with both α- and β-estrogen receptors. These estrogenic effects may be responsible for the beneficial actions of red clover in menopausal women. In addition there is evidence from a single study that suggests that red clover isoflavones influence opiate receptors responsible for body temperature and mood, providing actions that may also contribute to a reduction in menopausal symptoms.

Red clover preparations have also been clinically tested for their potential effects on symptoms of metabolic syndrome in postmenopausal women, with some studies reporting positive changes in lipid profiles, potential improvement in insulin sensitivity, and significant reduction of daytime blood pressure.

Red clover extract has also been investigated for its potential to reduce prostate cancer risk and was found to significantly increase the degree of apoptosis in subjects with low to moderate-grade prostate cancer. However, there was no effect on a variety of biomarkers of prostate cancer (e.g., PSA, Gleason score, etc.). Similar limitations are evident

with other endpoints, in that changes in biomarkers do not inherently confer clinical efficacy in terms of symptom relief or disease prevention or treatment.

The use of red clover preparations for menopause can be considered within the context of the desire to avoid the potential risks associated with conventional hormone replacement therapies (HRT). Further research is indicated to explore the comparative activities of the various individual isoflavones and the optimal dosage for the composite extract for various indications.

Medical Indications Supported by Clinical Trials

In menopause, moderately reduces hot flashes, depression, and cardiovascular risk, and positively affects lipid profiles. Likely most effective in equol-producers.

Medical Indications Supported by Traditional or Modern Experience

A variety of acute or chronic skin conditions (e.g., bite, burns, eczema, and psoriasis), asthma, bites, dry coughs (acute or chronic), eyewash, fever, gout, leucorrhea, nervous conditions, reduction of some cancerous growths, wounds. For most of these indications red clover was used both internally and topically as a douche, eyewash, fomentation, gargle, plaster or salve.

Actions

Alterative, anti-angiogenic, antioxidant, antiseptic, antispasmodic (mild), antitussive, emollient, expectorant, febrifuge, induces apoptosis of prostate cancer cells, mild nervine, mild nutritive tonic, phytoestrogenic, vulnerary.

Substantiated Structure and Function Statement

Helps to relieve symptoms associated with menopause, most notably hot flashes and mood.

Dosages

Powder:	4 g three times daily (BHP 1983).
Infusion:	4 g three times daily (BHP 1983).
Tincture (1:10, 45% ethanol):	1–2 mL three times daily (BHP 1983).
Fluidextract (1:1, 25% ethanol):	1.5–3 mL three times daily (BHP 1983).
Dry extract:	Equivalent to 40–80 mg isoflavones daily.

Discussion on Phytoestrogens

The term phytoestrogen describes a class of plants and plant compounds that are structurally and pharmacologically similar to endogenously produced estrogens (e.g., estradiol, estrone, estriol) but whose actions can be described as estrogenic, anti-estrogenic, and estrogen-independent. Phytoestrogens are present in a wide variety of fruits, vegetables, and whole grains and are especially plentiful in legumes, the botanical family to which red clover (and soy) belong. Phytoestrogens are metabolized by intestinal bacteria, absorbed, conjugated in the liver, circulated in plasma, and excreted in urine (see Pharmacokinetics). These metabolites often exhibit greater activity than their parent compounds. However, the ability to synthesize these metabolites is not equal in all individuals and may result in varying levels of activity and effects (Vergne et al. 2009).

Decades of epidemiological, clinical, and mechanistic data suggest phytoestrogens possess a host of beneficial albeit, modest, actions. First and foremost, phytoestrogens reduce symptoms and disease patterns that arise due to post-menopausal estrogen decline, including menopausal symptoms, cardiovascular disease, osteoporosis, skin deterioration, and mucosal dryness. Numerous reviews of these botanicals and their compounds have been published in recent decades (see Baber 2010; Guerro-Bosagna and Skinner 2014; Helferich et al. 2008; Messina 2010; Ososki and Kennely 2003; Patisaul and Jefferson 2010; Sirotkin and Harrath 2014; Tempfer et al. 2007; Virk-Baker et al. 2010, among others). All epidemiological study has been done with dietary consumption of phytoestrogens, not isoflavone supplements. Some studies report positive changes in biomarkers of disease but may not include actual endpoints in terms of reduction of incidence or severity and also has to be kept in mind.

The actions of phytoestrogens are complex and multifaceted but, due to their structural similarities to endogenous estrogens, can potentially affect all processes influenced by estrogens. Interpreting the findings of phytoestrogen research requires care in determining how these substances are best applied. As with most research, there is a significant amount of contradictory data, predominantly between in vitro and in vivo findings. In vitro research demonstrates that phytoestrogens partially elicit effects in a manner that is similar or identical to endogenous estrogens, including their propensity to stimulate cancer cell growth. Conversely, the preponderance of in vivo phytoestrogen research, including epidemiological, clinical, and mechanistic data, articulate numerous actions that are both cancer preventive and potentially, cancer reducing. Some actions associated with the in vivo use of phytoestrogens include antioxidant, antiproliferative, antimutagenic, and antiangiogenic effects. In vivo data also suggests benefit in reducing some symptoms of menopause, cardiovascular risk, and cancer risk, even in those with estrogen sensitive cancers, as well as general improvements in health and longevity.

While there is a plethora of data on the benefits of phytoestrogen intake, supported by both human and mechanistic data, caution is also required when interpreting the data between different phytoestrogen-containing botanicals and extrapolating findings to human health. From a purely botanical perspective, not all phytoestrogenic botanicals contain the same specific compounds in the same concentrations and ratios; therefore, different botanicals will act with varying levels of efficacy or be ineffective for specific

Table 12 Selective effects of phytoestrogen treatment on the cell cycle

Nature of isoflavone	Concentration	Cell type	Biological change	Reference
Genistein, glycitein, daidzein	0, 10, 25, and 50 µM each	Human bladder cancer cell line 253JB-V	a) Genistein, compared to other isoflavones, significantly inhibited the cell growth; b) Cell cycle arrest at G2/Mtransition	Singh et al. 2006
Genistein	2.5–40 µM	Cervical cancer cell lines CaSki and ME180	a) Dose-dependent inhibition of both cell lines; b) Cell cycle arrest at G2/Mtransition in ME180 cell line only	Yashar et al. 2005
Genistein alone or combined with radiation	15 µM genistein alone or 3 Gy radiation alone or 15 µM genistein + 3 Gy radiation	Prostate cancer cell line PC-3, human breast cancer cell line BR231 human renal cancer cell lines KCI-18 and RC-2	Genistein combined with radiation compared to either treatment alone: a) Significantly inhibited cell growth in all cell lines; b) Significantly greater G2/Mcell cycle arrest; c) Downregulation of cyclin B1; d) Upregulation of P21WAF1 pretreatment of cells with 30 µM genistein followed by 3 Gy radiation significantly decreased NF-kB DNA binding	Raffoul et al. 2006
Genistein	0–370 µM	Human renal carcinoma cell lines SMKT R-1, R-2, R-3, and R-4	Dose-dependent inhibition of growth and the 100 µg/L (370 µM) dose resulted in a time-dependent inhibition in all four cell lines	Sasamura et al. 2004

Source: Virk-Baker et al. 2010.

clinical endpoints. Physiologically, ERα and ERβ receptors are variously expressed in different tissues, and may even express differently at different stages of development (fetal development, puberty, and maturity) and so the effects of phytoestrogens on estrogen receptors can vary with developmental period and are therefore exceedingly complex. Though generally of benefit and safe, varying clinical endpoints may benefit from one set of phytoestrogens over another, or conversely, and in rare cases, may be best avoided, a factor further complicated by genetic polymorphisms.

Endogenous Estrogens and Phytoestrogens

Estrogens exert their affect through activation of two types of receptors, ERα and ERβ, which are concentrated in different tissues. The relative distribution of ERα and ERβ mRNA is predominantly determined in animal models (Kuiper et al. 1998). ERα mRNA is highly expressed in tissues of the epididymis, testes, ovaries, kidneys, and adrenals, in the developing fetus, and in the uterus (Brandenberger et al. 1997). Moderate amounts of ERα are also located in the prostate, bladder, hypothalamus, liver, and thymus (Ascenzi et al. 2006; Liu and Shi 2015). Significantly high amounts of ERβ mRNA are present in the prostate, ovaries, the central nervous, cardiovascular, and immune systems, gastrointestinal tract (Ascenzi et al. 2006; Brandenberger et al. 1997), is highly expressed in the epidermis, blood vessels, and dermal fibroblasts of the skin (Thornton et al. 2003), in fetal development, ovaries, testes, adrenals, and spleen. ERβ mRNA is only moderately expressed in the uterus (Brandenberger et al. 1997) and is highly expressed in bladder cancer cells (Shen et al. 2006). Both ERα and ERβ receptors express in the brain (Weiser et al. 2008). Additionally, while exceptional polymorphisms exist, ERα receptors, generally, are correlated with breast tumorigenesis, while ERβ influences cellular apoptosis (Covaleda et al. 2008). ERβ expression is also generally protective against colon, prostate, and endometrial cancers and protects against bone loss and heart disease (Ascenzi et al. 2006). Because of the dualistic and opposing activity of α- and β-receptors in terms of tumorigenesis and apoptosis, respectively, the ratios of these two may play a role in whether phytoestrogens elicit or inhibit cancer cell proliferation. Both quercetin and genistein have a greater affinity for ERβ and for activating ERβ gene expression. In human breast cancer cell lines (T47D-ERβ), this results in a decrease in estradiol-induced cellular proliferation (Sotoca et al. 2008).

Estradiol binds equally to both ERα and ERβ receptor types; estrone binds preferentially to α-receptors; estriol binds preferentially to β-receptors. While endogenous estrogens are steroidal sex hormones, phytoestrogens are nonsteroidal, a significant difference between the two that is partly responsible for the benefits and relative safety of phytoestrogens. Steroidal hormones act directly as hormones, are fat-soluble, can pass through the cellular membranes of target tissues (Heffner and Schust 2010), and can initiate or contribute to tumorigenesis (Singh and Kumar 2005). Conversely, phytoestrogens are water-soluble, cannot pass into cells, and instead, mimic true estrogens exerting their affect through the activation of secondary messengers, rather than via direct steroidal activity. Additionally, phytoestrogens (e.g., genistein, equol) typically are less potent estrogen receptor activators than endogenous estrogens (Collins et al. 1997) and the synthetic estrogen diethylstilbestrol (Mueller et al. 2004), specifically, 100 to 9,000 times less potent (Collins et al. 1997). Conversely, circulating phytoestrogens can exceed those of endogenous estrogens by 1,000-fold

(Messina 2010), underscoring why a physiological effect can be expected and highlighting the net anti-estrogenic effect through competitive receptor binding of weak versus stronger estrogens.

Phytoestrogens can bind to both ERα and ERβ receptor types but preferentially bind to ERβ receptors (Maximov et al. 2013; Minutolo et al. 2011). They possess both estrogen agonist and antagonist (Mueller et al. 2004) effects depending on the concentration and target tissue affected. Because of this dual action, phytoestrogens are sometimes referred to as selective estrogen receptor modulators (SERMs), a term also applied to the conventional medications raloxifine and tamoxifen. Some isoflavones, including those in red clover, additionally exhibit binding affinity for androgen (AR) and progesterone (PR) receptors (Pfitscher et al. 2008) and act as inhibitors of aromatase and 3β- and 17β-hydroxysteroid dehydrogenase, enzymes involved in the biosynthesis of androgens into estrogens (Krazeisen et al. 2001; Moon et al. 2006). Besides their effects on estrogen binding and synthesis, phytoestrogens have several other mechanisms of action that contribute to their overall effects (see *Non-Estrogenic-Like Effects of Phytoestrogens* below).

Phytoestrogen Compounds

In general, the primary classes of compounds associated with phytoestrogen activity include chalcones, flavonoids (flavones, flavonols, flavanones, isoflavonoids, pterocarpans), coumestans, lignans, prenylpropanoids, and stilbenoids, among others (Dixon 2004; Michel et al. 2013; Sirotkin and Harrath 2014). The most studied of the phytoestrogen plants is soy (*Glycine max*) due to its high concentration of isoflavones and documented relationship in reducing the incidence of cancers and heart disease. These benefits are predominantly reflected in numerous epidemiological studies conducted in various countries including China (Zhang et al. 2003) and Japan (Nagata 2000). The Japanese diet typically provides approximately 20 to 80 mg/d of isoflavones, as compared to North American diets that deliver below 1 mg/d. The benefits of soy intake led the Food and Drug Administration (FDA) to approve of a claim that 25 g/d of soy protein can reduce the risk of heart disease.

The most studied of the specific phytoestrogen compounds are daidzein, genistein, formononetin, biochanin A, and glycitein (all of which are contained in red clover), and their metabolites, most notably equol (4'7'-isoflavandiol), a metabolite of daidzein synthesized to greater or lesser degrees in the intestines (Wang et al. 2005). There are two forms of equol, (*S*)-equol and (*R*)-equol (Setchell and Clerici 2010); the former preferentially activates ERβ receptors (Mueller et al. 2004; Wang et al. 2005).

Confirmed Benefits of Phytoestrogens

Initial interest in the health benefits of phytoestrogens was stimulated by epidemiological studies showing that dietary consumption of isoflavones (initially) as reflected in Asian diets) was a prominent dietary factor correlated with a reduced risk of breast (for a review see Wu et al. 2013), endometrial (Bandera et al. 2009; Horn-Ross et al. 2003), ovarian (Bandera et al. 2011), and prostate cancer (Yan and Spitznagel 2009) and heart disease (e.g., Kokubo et al. 2007; Liang et al. 2009; Zhang et al. 2003, among others). Since then, other benefits and putative benefits have been the focus of phytoestrogen research including the symptomatic relief of menopausal symptoms, preservation of post-menopausal bone density (Marini et al. 2008), cognition and mood (Casini et al. 2006), skin health (Patriarca et al. 2013), and dry eyes (Scuderi et al. 2012), in post-menopausal women. Mechanistic data also support many of the benefits reported in the epidemiological and clinical literature.

Non-Estrogenic-Like Effects of Phytoestrogens

In addition to the effects of phytoestrogens in mimicking estrogenic activity through estrogen receptor binding, there are numerous other physiological mechanisms elicited by phytoestrogens. Most of these effects have been established in vitro and animal studies and may or may not be clinically relevant, though some provide potential explanations for observed clinical effects. Phytoestrogens elicit a broad variety of activities that contribute to their putative anticancer effects and include: inhibition of inflammation, angiogenesis, and metastases, all of which have been demonstrated in various in vivo tumor models (Virk-Baker et al. 2010); act as protein tyrosine kinase inhibitors (e.g., the epidermal growth factor receptor) (Akiyama et al. 1987); possess antioxidant activity (Campbell et al. 2004); inhibit tumor necrosis factor alpha (TNFα) (Rice et al. 2006), act as PPAR agonists (Jungbauer and Medjakovic 2014; Mueller and Jungbauer 2008); inhibits DNA topoisomerase II activity (Markovits et al. 1989); suppresses angiogenesis (Fostis et al. 1995); induces breast cancer cell apoptosis (Li et al. 1999a; Li et al. 1999b; Pagliacci et al. 1994); and downregulates HER2 (Katdare et al. 2002; Li et al. 1999b), and ERα expression (Chen et al. 2003; among others). A final, under-investigated action of flavonoids in general, and genistein specifically, is that they are potent non-competitive inhibitors of sulfotransferase 1A1 (or P-PST), which activates phase II detoxifying enzymes, such as UDP-glucuronyl transferase, glutathione S-transferase, and quinone reductase that results in the detoxification of carcinogens (Moon et al. 2006).

Caution in Interpretation of Data

While, considerable investigation has been undertaken regarding the use of conventional SERMs in the treatment of cancers (e.g., tamoxifen), little comparative research has been done to determine the optimal application of phytoestrogens, especially in various cancers, though the preponderance of data show a net cancer protective effect correlated with phytoestrogen consumption. Perhaps most importantly, the cancer protective effects of phytoestrogens are correlated with the actions of phytoestrogen metabolites (e.g., daidzein, equol, genistein, *O*-desmethylangolensin). Most notably, equol is a metabolite of daidzein, the conversion of which takes place in the intestines.

Approximately 50% of Asians and 25% of Westerners are

equol producers. Equol production is associated with lower concentrations of testosterone, androstenedione, dihydroxyepiandrosterone (DHEA), DHEA sulfate, and higher levels of SHBG, regardless of isoflavone consumption. Female equol producers tend to have lower midluteal phase plasma estrone, estrone sulfate, and progesterone and higher FSH levels versus nonproducers. Of note, the ability to produce equol is temporarily lost after antibiotic exposure (Messina 2010). One meta-analysis demonstrated a reduction in breast cancer risk due to soy consumption among pre- and post-menopausal women but not among women in western countries (Chen et al. 2014a).

Another aspect of phytoestrogen intake that has not been fully appreciated but for which there is a suggestion of evidence, is that most epidemiological data supporting a cancer preventive effect suggest that life-long (pre-pubescent/adolescent) exposure to these compounds as part of the diet confers the greatest benefit (28–60%) (Hilakivi-Clarke et al. 1999; Messina and Hilakivi-Clarke 2009; Messina and Wu 2009; Peng et al. 2009). In contrast, results of isoflavone-exposure studies of adults are mostly negative regarding a cancer protective effect. Of significant importance regarding safety, phytoestrogen exposure (demonstrated with soy) does not appear to increase the risk of cancer in vivo, even in those with estrogen sensitive cancers or a history of breast cancer (Guha et al. 2009; Hooper et al. 2009; Kang et al. 2010; Messina and Loprinzi 2001; Shu et al. 2009). At least one study (Kang et al. 2010) suggests that consumption of soy isoflavones decreased the incidence of breast cancer recurrence. Another three-year study of women (n = 284) with a familial history of breast cancer (first-degree relative) specifically with red clover supplementation (40 mg/d), revealed no increased breast cancer risk or changes in endometrial and breast tissues (Powles et al. 2008). Other human studies demonstrate a lack of a significant effect of phytoestrogens on markers of reproductive cancers such as endometrial or vaginal thickening (Nikander 2005), increased breast density (Frankenfeld et al. 2004; Marini et al. 2008), or increased cancer risk in post-menopausal women (Touillaud et al. 2006) (see Phytoestrogen Discussion and Carcinogenicity below).

Perhaps most significantly from a clinical perspective is that proposed benefits of phytoestrogens are often based on improvements in biomarkers that are surrogates for reducing risk factors rather than findings of actual reduced symptom or disease incidence. For example, phytoestrogens are associated with improvements in bone metabolism and density but there are no data demonstrating a reduction in fractures. Phytoestrogens are similarly correlated with a reduction in a number of cardiovascular risk factors such as improvements in endothelial cells, vascular smooth muscle, and extra-cellular matrix, decreased arterial stiffness, and antiatherosclerotic effects, but no definitive reduction in the incidence of cardiovascular disease (Gencel et al. 2012; Gil-Izquierdo et al. 2012; Wuttke et al. 2002). Thus, care is needed in interpreting data and attempting to extrapolate it into either benefit or risk in humans.

Summary

While the totality of data, clinical, epidemiological, and mechanistic, support a general beneficial effect of phytoestrogens in reducing menopausal symptoms, maintaining bone density after menopause, and exerting an anticancer and cardiovascular protective effect, among other general health benefits, the findings are mixed and, when positive, are weak or moderate. Therefore, women may not obtain the relief sought by taking phytoestrogens. In some cases (e.g. bone density and heart disease), putative benefits are extrapolated from surrogate biomarkers of disease rather than observed reductions in disease incidence. Botanically, not all phytoestrogens are found in the same quantities and ratios, and so effects of phytoestrogenic plants will vary widely. Physiologically, even greater variation of effects can be observed between those who, for example, are equol- versus non-equol producers.

The multiplicity of effects associated with phytoestrogens is the key to their putative benefit. Collectively, this multiplicity of actions differentiate phytoestrogens from endogenously produced estrogens in inhibiting cell cycle events and provide mechanistic support for the putative cancer preventive effects of phytoestrogens demonstrated in a variety of preclinical models (Virk-Baker et al. 2010, among others) and expressed in epidemiological studies (Nagata et al. 1998; Zhang et al. 2003, among others). This collective of actions also help to clarify that despite the typical characterization of this class of plants and compounds as phytoestrogens, the effects are more accurately anti-estrogenic in net effect. In this regard, phytoestrogens have been described as an "ideal" category of drugs for reducing the risk of estrogen dominant breast cancers (Ascenzi et al. 2006).

Therapeutically, the weak activity of phytoestrogens (100–9000-fold less potent; Collins et al. 1997), relative to endogenous or supplemental estrogens in the form of conventional hormone replacement therapy (HRT), explain why only moderate benefits are observed in clinical studies regarding menopausal symptoms. Conversely, the relative weakness of phytoestrogens is also the reason for the safety of phytoestrogens, not only as an alternative to conventional HRT with other integrative therapies (diet, weight bearing exercise, etc.), but as a general source of health-promoting phytochemicals, not only for cancer, but for heart disease and osteoporosis. Thus, the weak to moderate benefits associated with phytoestrogens can be weighed against conventional therapies that may have a greater adverse effects profile, which are significant.

SAFETY

When consumed within normal consumption and supplementation patterns, red clover is a very safe botanical. Historically, red clover was widely consumed as a tea and sometimes eaten, and is widely used as a part of animal feeds. Adverse effects in animals can occur if moldy red clover is fed to animals. Various molds are implicated in these effects. The mold slaframine, which causes slobbers, is

Table 13 Human studies investigating the effects of phytoestrogens on sexual development

Study Population	Method	Findings	Reference
Puerto Rican, girls aged six mo to eight yr (130 PT cases, 130 controls)	Cross-sectional study Assessment of breast bud	Positive association between PT and consumption of soy-based formula. Note: Puerto Rico has a rate of thelarche that is 18.5 times higher than in Minnesota (Van Winter, et al. 1990); follow-up studies specifically in Puerto Rico have not identified phytoestrogen exposure as a contributing factor (Aviles et al. 2014)	Freni-Titulaer et al. 1986
Israel, female infants (total 694, soy formula feeding 92)	Cross-sectional study Assessment of breast bud	Positive association between PT and consumption of soy-based formula	Zung et al. 2008
US, infants (35 girls)	Follow-up study Assessment of breast bud, vaginal wall cells	Girls fed soy-formula showed re-estrogenization at six months in vaginal wall cell maturation index	Bernbaum et al. 2008
US, females aged 20-34 yr (128 fed soy, 268 fed cow milk formula in infancy)	Retrospective cohort study; assessed pubertal timing by telephone interview	No difference in pubertal timing between groups	Strom et al. 2001
US, New York girls aged nine yr (192)	Cross-sectional study Assessment of Tanner stages	A negative trend for urine daidzein and genistein levels with breast development	Wolff et al. 2008
German, girls (119)	Cohort study Assessment of dietary intake, breast development	A negative trend for dietary isoflavones with breast development in girls; No association for urinary isoflavone levels with pubertal markers	Cheng et al. 2010
UK, girls (2,920)	Prospective study Assessed age at menarche by annual questionnaire	Girls fed soy-formula in early infancy have an increased risk of menarche	Adgent et al. 2012
Korea, girls (108 CPP cases, 91 controls)	Case-control study Assessed Tanner stage, bone age, GnRH stimulation test	A positive association between CPP risk and high serum isoflavone level	Kim et al. 2012
US, males aged 20–34 yr (120 fed soy, 295 fed cow milk formula in infancy)	Retrospective cohort study; assessed pubertal timing by telephone interview	No difference in pubertal timing between groups	Strom et al. 2001
German, boys (108)	Cohort study Assessment of dietary intake, gonadal development	No association with gonadal development in boys; no association for urinary isoflavone levels with pubertal markers	Cheng et al. 2010
UK, boys (total 7,928,51 hypospadias cases)	Prospective study; Assessed life style and dietary practices by questionnaire during pregnancy	Increased risk of giving birth to a boy with hypospadias in vegetarian mothers compared with omnivores	North and Golding 2000

PT = premature thelarche; CPP = central precocious puberty. Source: Modified from Kim et al. 2012.

typically detectable as rust-like spots on the upper side of the leaves or black spots on the under side of leaves (black botch disease) (Murphy 2016). Sweet clover poisoning associated with *Melilotus officinalis* and *M. alba* can cause hemorrhagic disease as molding can result in a transformation of coumarins contained in the plant to dicoumarol (see Red Clover and Coumarins below). Adverse effects from moldy clover have not been reported in humans.

Concerns regarding phytoestrogen consumption that have been articulated in the literature include male feminization, increased breast cancer risk, thyroid impairment, infertility, and abnormal development. Secondary concerns include impaired cognition and endometrial cancer. None of these adverse effects have been associated with red clover consumption and are addressed below. The European Food Safety Authority (EFSA 2015) was asked to deliver a scientific opinion on the possible association between the intake of isoflavones from food supplements and harmful effects on the mammary gland, uterus, and thyroid in peri- and postmenopausal women. According to this review, the human data did not support an increased risk of breast cancer from observational studies nor of an effect on mammographic

density nor on proliferation marker Ki-67 expression in interventional studies. No effect was found on endometrial thickness and histopathological changes in the uterus with up to 30 months of supplementation with 150 mg/d of soy isoflavones. Similarly, levels of thyroid hormones were not changed following isoflavone supplementation. After 60 months, some non-malignant histopathological changes were reported.

Care must be taken in interpreting red clover data, especially in vitro and animal data, as many pre-clinical findings are completely contrary to what is demonstrated in humans, partially due to metabolic differences between the species and partly due to excessively high doses used in animal studies relative to human exposure, a concern voiced in formal toxicological reviews (COT 2003). For this reason, this safety review focuses primarily on formal human clinical trials.

Adverse Reactions

Based on a number of reviews and meta-analyses of clinical trials, adverse events reported across numerous clinical trials of red clover products are rare, minor, and similar between the placebo and red clover groups (e.g., Coon et al. 2007; Lethaby et al. 2007; Low Dog 2005, among others). Most reviewers note no apparent evidence of adverse events during short-term use of red clover, and long-term studies were lacking. Based on a meta-analysis of 174 randomized controlled trials, there was a moderate increase in gastrointestinal side effects. Otherwise, the side effects profile between phytoestrogen intake and control groups was similar (36.7% and 38.0%, respectively) (Tempfer et al. 2009). Rates of vaginal bleeding, endometrial hyperplasia, endometrial cancer, and breast cancer with consumption of phytoestrogens are not increased. Doses of products in the included studies ranged from 40 and 82 mg/d for up to one year (Coon et al. 2007; Lethaby 2007). According to another systematic review, gastrointestinal symptoms were generally the most common adverse events experienced in both isoflavone and placebo groups (Nelson et al. 2006). No case reports of adverse reactions were identified.

Interactions

In a meta-analysis of five clinical trials, no clinically relevant negative in vivo interactions were reported (Coon et al. 2007). In a systematic review of 40 RCTs, 11 uncontrolled trials, and 80 observational studies that included soy, red clover, and isoflavones, no increased risk for breast cancer or breast cancer recurrence was evident (Fritz et al. 2013). Reviews of other human studies report similar findings (see Caan et al. 2011; Guha et al. 2009; Wu et al. 2007). Caan et al. (2011) reported a trend toward decreased risk of death among tamoxifen users with intake of total isoflavones (≥6.3 mg/d) and perhaps of greater relevance, among women with ER+ or PR+ status. In a pooled investigation of 9,515 breast cancer survivors, high soy food intake after cancer diagnosis was associated with a statistically significant reduction of breast cancer recurrence and a non-significant reduced risk of all-cause mortality. Similarly, tamoxifen users who had higher isoflavone intakes had a reduced risk of both all-cause mortality and breast cancer recurrence, compared to women who did not use tamoxifen and consumed the lowest amount of soy food (Caan et al. 2011).

A number of human studies similarly found no negative interaction with aromatase inhibitors (Caan et al. 2011; Guha et al. 2009; Shu et al. 2009). Animal data regarding the effects of individual isoflavones and interaction with tamoxifen are mixed with some studies (e.g., Ju et al. 2002) reporting that genistein inhibits the antiestrogenic effect of tamoxifen and others showing isoflavones synergistically enhance the efficacy of tamoxifen (Fritz et al. 2013; Gotoh et al. 1998; Mai et al. 2007a). Mechanistic studies suggest that genistein may sensitize ER+ and HER2-overexpressing breast cancer cells to treatment with tamoxifen, a significant finding considering that approximately 40% of estrogen-dependent breast tumors do not respond to tamoxifen treatment (e.g., Mai et al. 2007b). In a similar manner, in vitro and in vivo (xenograft model) research demonstrated that genistein (10 µM) sensitizes bladder cancer cells to chemotherapeutic treatment with hydroxycamptothecin (Wang et al. 2013). Whether this has any clinical relevance in human bladder cancers is not known.

In rats, no effect on pharmacokinetics of tamoxifen and 4-hydroxytamoxifen by multiple doses of administration of red clover (45 mg/kg/d) 15 days prior to tamoxifen (10 mg/kg/d) was observed. Other CYP substrate interactions were tested and similarly found to be unaffected by red clover. The extract yielded the following: formononetin 1.59 mg/g, biochanin A 2.03 mg/g, genistein 0.94 mg/g, and daidzein 1.36 mg/g (Rama-Raju et al. 2015).

An ethanolic extract of a commercial red clover preparation inhibited the drug-metabolizing isoenzyme CYP3A4 in a fluorometric microtiter plate assay (in vitro) (Budzinski et al. 2000). Daidzein (K_i = 3.7 µM) exhibited competitive inhibition of CYP1B1 7-ethoxyresorufin O-deethylase activity, and genistein (K_i) 1.9 µM) exhibited mixed inhibition. According to these researchers, biochanin A and/or formononetin may exert anticarcinogenic effects directly by acting as competitive substrates for CYP1B1 or indirectly through their metabolites daidzein and genistein, which inhibit CYP1B1 (Roberts et al. 2004).

Reproductive And Developmental Effects

Information on the safety of red clover during pregnancy is limited. Pregnant or nursing women are advised to discuss the possible benefits and risks of botanical medicines with a qualified healthcare practitioner prior to use. Anti-infertility effects of soy isoflavones have been reported in animals but the concentration of circulating hormones from the animal diet are too great to realistically be achieved in humans. Negative reproductive effects observed in sheep ("clover disease") is due to a very high isoflavone exposure. Similar effects are observed in captured cheetahs due to the animal's inability to glucoronidate isoflavones (Hooper et al. 2009).

Effects on Circulating Hormones

Most concern regarding phytoestrogen consumption arises from their potential to alter hormone status, most notably

in women. Changes in mammographic breast density and endometrium thickening due to circulating hormones are two primary models used as predictors of carcinogenicity. In a single study, regarding red clover specifically, 177 women (aged 49–65) consumed a red clover tablet containing 26 mg biochanin A, 16 mg formononetin, 1 mg genistein and 0.5 mg daidzein), or placebo for 12 months. There were no significant effects on estradiol, FSH, or LH (assessed only in postmenopausal women), and no increase in mammographic breast density (Atkinson et al. 2004c). In women aged 35–70 with at least one first-degree relative with breast cancer, administration of 40 mg/d of a red clover isoflavones for three years did not result in any significant differences in breast density, endometrial thickness, or follicle-stimulating hormone levels, as compared to placebo (Powles et al. 2008). In a meta-analysis of five red clover studies (Lethaby et al. 2007), there was no evidence of estrogenic stimulation of the endometrium. Similar findings have been reported in soy isoflavone studies. For example, in an assessment of 47 soy studies, no changes in estradiol, estrone, or serum hormone binding globulin (SHGB) were reported. In an analysis of 10 of the 47 studies, increased soy/isoflavones intake was correlated with an increase in menstrual cycle length by 1.05 days. In post-menopausal women, there were no significant effects on estradiol, estrone, or SHBG, although there was a small statistically non-significant increase in total estradiol with soy or isoflavones across 21 of the studies (Hooper et al. 2009). In one study with daidzein, postmenopausal women (n = 58) consumed a soy bar containing 83 mg/d daidzein or soy nuts containing 10 mg/d daidzein. In this study, women found to be equol-producers had an average of 39% lower percent mammographic density than non-equol producers (Frankenfeld et al. 2004). Whether this finding results in a clinically relevant benefit, is not known but provides some evidence of a lack of risk based on this marker. In another study, long-term exposure to genistein (54 mg/d) similarly elicited no effects on increasing breast density (Marini et al. 2008).

Conversely, in a non-controlled clinical study of postmenopausal women administered 80 mg/d of red clover–derived isoflavone extract for six months, some changes in endometrial activity but no changes in endometrial thickness were observed. Of the 32 study participants, six presented vaginal bleeding and three presented endometrial alteration as compared to the initial exams, two developed endometrial cell proliferation, and one developed endometrial hyperplasia (Wolff et al. 2006).

In a study of amniotic fluid samples in women between weeks 15 and 23 of pregnancy, isoflavonoids were detected in 92% of samples. The isoflavonoids daidzein, genistein, formononetin, biochanin A, and coumestrol were detected (Foster et al. 2002) demonstrating fetal exposure. Specifically regarding genistein, placental metabolizing enzymes conjugate a small fraction of genistein into the glucuronide/sulfate form, which is devoid of estrogenic action (Balakrishnan et al. 2010). Based on epidemiological studies in countries with relatively high isoflavone consumption patterns (25–50 mg from soy in the Japanese diet) that are similar to supplementation values (40–80 mg), no negative reproductive effects are to be expected. In a review of the effects of genistein on reproduction and development, the most highly exposed adult population of Japan (0.43 mg/kg) was approximately 10-fold less than the no-effect levels determined in rodent studies (Barrett 2006).

Animal and In Vitro Data

Effects on Reproductive Tissues

In ovariectomized rats administered 250, 500, or 750 mg/d of a red clover extract (15% isoflavones) for 21 days, a dose-dependent increase in uterine weight and differentiated vaginal cells were observed at the two higher doses, but no stimulation of cell proliferation was observed in the mammary glands. Neither antiestrogenic nor additive estrogenic properties were observed in any of the tissues studied (Burdette et al. 2002).

In vitro assays of red clover extracts in endometrial cells and MCF-7 (estrogen receptor–positive) breast cancer cells, differential estrogenic activity was observed in the endometrial cells whereas non-differential activity was observed in the MCF-7 cells, indicating the significance of the type of bioassay used to determine the estrogenic activity of red clover (Booth et al. 2006b).

An extract of a red clover isoflavone preparation increased MCF-7 breast cancer cell proliferation rates (Bodinet and Freudenstein 2004). A standardized red clover isoflavone extract (9% isoflavones by dry weight) showed an affinity for both estrogen receptor (ER) α and β with a significantly stronger affinity for the ERβ receptor in a yeast two-plasmid system (Dornstauder et al. 2001).

The addition of red clover (75 g/kg yielding biochanin 0.3%, formononetin 0.27%, daidzein 0.01%, and genistein 0.01%) to the diet of pregnant rats (starting day seven of gestation to day 21) resulted in a non-significant reduction in body weight and significant increases in ovarian and uterine weight (Yatkin and Daglioglu 2011).

A red clover–derived extract of 30% isoflavone aglycones was shown to bind to mu- and delta-opiate receptors in Chinese hamster ovaries (Nissan et al. 2007).

Effects on Thyroid

There are reports of the potential interaction between isoflavones and the synthesis of thyroid hormones. In the EFSA (2015) review, 11 human controlled randomized trials that reported effects of isoflavones administration on some thyroid-related endpoints were identified. In total, 925 subjects were allocated to isoflavones. Only serum TSH was measured in all 11 studies, T4 and/or fT4 was assayed in eight studies, T3 and/or fT3 was assayed in five studies, thyroid autoantibodies in two studies, and thyroxine-binding globulin (TBG) or thyroglobulin in one study. In none of the studies was a clinically relevant effect on the thyroid detected. Although the studies have some flaws (thyroid function not the primary endpoint, sample size calculation not given, low power to detect changes) the review concluded that administration of food supplements containing isoflavones is not associated with clinically relevant changes

in thyroid function (hypo- or hyperthyroidism) in peri- and post-menopausal women.

Carcinogenicity

There is no in vivo evidence to suggest that red clover specifically, or phytoestrogen supplementation generally, increases cancer risk in humans. In contrast, there is a plethora of data demonstrating cancer-preventive effects of phytoestrogens overall. Numerous clinical studies and systematic reviews have specifically investigated the potential for phytoestrogens to initiate or contribute to carcinogenicity in various populations (childhood, healthy adults, breast cancer survivors, individuals with breast cancer risk) but no such risk is evident (Fritz et al. 2013; Nechuta et al. 2012: Touillaud et al. 2006). In a review of 26,868 premenopausal women (mean age, 47 years) reported that median dietary intake of total phytoestrogens was 1,101 µg/d, mostly consisting of plant lignans. Similarly, no association between dietary phytoestrogen intake and breast cancer risk in premenopausal women and ER+/PR+ status was found (Touillaud et al. 2006).

In human studies, varying data on the putative estrogenic activity of red clover has been reported, raising concerns of increased carcinogenicity with increased phytoestrogen exposure. No such negative effects have been observed with red clover supplementation, in contrast to estradiol supplementation, much of the difference that is explained by differences in the manner in which phytoestrogens exert their effects versus endogenous estrogens (see Phytoestrogen Discussion). Some studies show no effect of red clover supplementation on endometrial thickness or vaginal cytology (Baber et al. 1999b; Clifton-Bligh et al. 2001; Hale et al. 2001), indicating a lack of estrogenic activity. Other studies show a decrease of endometrial thickness (Imhof et al. 2006) or a significant improvement in vaginal cytology (Hidalgo et al. 2005). No studies report an increase in endometrial thickening (see Table 11).

No significant differences in vaginal cytology, endometrial thickness, serum estradiol, follicle-stimulating hormone, or sex hormone-binding globulin were observed after administration of 40 mg/d of a red clover–based isoflavone extract (Baber et al. 1999b). No increase in endometrial thickness was observed after administration doses up to 85.5 mg/d red clover isoflavone preparation for six months (Clifton-Bligh et al. 2001). A decrease in endometrial thickness and increase in plasma testosterone levels were observed in postmenopausal women after administration of 80 mg/d red clover–derived isoflavones for 90 days (Imhof et al. 2006). After administration of 50 g/d of a red clover–derived isoflavone extract to perimenopausal women for three months, no evidence of an antiproliferative effect in the Ki-67 proliferative marker of endometrial biopsies was found (Hale et al. 2001). In postmenopausal women administered 80 mg/d of a red clover isoflavone supplement for 90 days, significant improvement in all vaginal cytology indices (karyopyknotic index, cornification index, maturation index), as compared to placebo, was observed (Hidalgo et al. 2005).

A systematic review of 131 studies on soy, red clover, and isoflavones that included 40 randomized controlled trials (RCTs), 11 uncontrolled trials, and 80 observational studies found no evidence of harm from use of soy with respect to risk of breast cancer or recurrence, based on long term observational data. Human trials generally showed that soy does not increase circulating estradiol or affect estrogen-responsive target tissues. Prospective data of soy use in women taking tamoxifen similarly did not indicate increased risk of breast cancer recurrence (Fritz et al. 2013).

A review of studies on red clover and soy-derived isoflavones concluded that 2 mg/kg/d was a safe dose of isoflavones for most populations (Barnes 2003). A second review of studies on red clover and soy-derived isoflavones indicated that 40–50 mg of isoflavones is recommended as daily dose. This recommendation is based on the daily intake of isoflavones in a traditional Japanese diet (Beck et al. 2005).

Together, the totality of data demonstrates a lack of clinically relevant estrogenic activity and carcinogenic potential of red clover, soy, and individual isoflavones.

Effects on Hormone Sensitive Cancers

Due to the putative estrogenic activity of phytoestrogens, there is a theoretical concern that phytoestrogens may exert a carcinogenic effect on hormone sensitive tumors. The existing data does not support a clinically relevant interaction between phytoestrogens and estrogen-sensitive tumors. Much of the available data is from studies of soy. While such data cannot be automatically extrapolated to other phytoestrogen-containing botanicals, such as red clover, it does inform understanding regarding this potential. Imhof et al. (2008) demonstrated that isoflavones exhibited only a minor activity as an agonist on the ERα receptor of a MCF-7 breast cancer cells in an estrogen-free culture that resulted in cell proliferation. When using a culture medium with synthetic and metabolized estrogen (relatively reflecting the serum estrogen level of a menopausal woman) the isoflavones acted as a competitive antagonist to serum estrogen protecting the ERα receptor from the endogenic serum estrogen activity of a peri- and menopausal woman which resulting in decreased MCF-7 breast tumor cell proliferation. This partly contributes to the understanding of why phytoestrogens exhibit a cancer inhibitory effect.

The soy isoflavone intake of 1,954 female breast cancer survivors was assessed and risk of breast cancer recurrence measured. There was a trend (P =0.08) for cancer recurrence reduction correlated with increasing quintiles of daidzein and glycetin intake among postmenopausal women and tamoxifen users. Of women using tamoxifen, a 60% reduction in breast cancer recurrence was observed in those with the highest (>1453 µg/d) versus lowest (<7.7 µg/d) intake of daidzein (Guha et al. 2009).

Toxicology

A formal repeated 28-day oral toxicology study of a red clover extract containing approximately 18% isoflavones was performed in rats (Frigo et al. 2001). Parameters tested included hematological, clinical chemistry, urinary analysis, and necropsy to determine if any damage to internal organs

occurred. No significant chronic oral toxicity was observed even at the limit-test dosage of 1,000 mg/kg/d.

In ovariectomized sheep, feeding with 3.5 kg/d of red clover silage for 14 days as the sole source of food (81–95 mg/kg/d total phytoestrogens) resulted in increased plasma concentrations of T3 and T4 and increased thyroid follicle size as compared to sheep fed hay (Madej et al. 2002). Isoflavones are reputed to interact with the synthesis of thyroid hormones. No such effects have been reported for red clover consumption. As noted above, the EFSA (2015) review noted that thyroid hormones were not changed following isoflavone supplementation.

Contraindications

None known.

Precautions

None known. There have been theoretical concerns raised regarding the presence of coumarins (common in *Fabaceae*) and the potential for bleeding events, as well as the potential for hepatotoxicity. Risk of bleeding events is not relevant to humans. The amount of coumarin associated with liver toxicity is magnitudes higher than is achievable with normal or even excessive red clover supplement use and there have been no reports of hepatotoxicity associated with red clover consumption (see discussions below).

Red Clover and Coumarins

Red clover is mistakenly cited as a potential anticoagulant due to the presence of coumarins. Considerable confusion exists between coumarins and the anticoagulant drug coumadin (sometimes referred to as coumarin) (Abebe 2002; Booth et al. 2004; Fugh-Berman and Kronenberg 2001; Heck et al. 2000). Generally speaking, naturally occurring plant coumarins are inactive as anticoagulants and are abundant in common edible plants including apricots, black currants, cherries, cinnamon, and strawberries. Conversely, while generally inactive in humans, plant coumarins are converted to the active form, dicoumarol (4-hydroxycoumarin) (DeSmet 1992) in the guts of ruminants, which has been associated with bleeding events, predominantly in sheep and cattle (Bye and King 1970; Link 1959). This conversion does not take place in human digestion. Moreover, relatively high concentrations of dicoumarol of 25–200 mg/d are needed to produce a clinically relevant anticoagulant effect (Booth et al. 2004). A dosage of a standard red clover extract of 400 mg/d yields approximately 0.28 mg/d of total coumarins. A screening of red clover identified five simple coumarin compounds in red clover, one (scopoletin with in vitro anticoagulant activity, one (daphnoretin) with in vitro procoagulant activity, and three (xanthtoxol; 8-hydroxypsoralen, fraxidin, coumesterol) with no activity reported. No changes in bleeding or blood clotting were observed in women after 30 days of red clover supplementation. No change in prothrombin time or INR time was found after administration of 400 mg/d of red clover extract to postmenopausal women for 30 days (Booth et al. 2004).

There is a possibility that a conversion from inactive coumarins to active dicoumarol can occur in plants through fermentation if plant material is not dried and stored properly but no reports of bleeding events in humans has been reported.

Conversely, there is evidence of platelet antiadhesion activity associated with different clovers, including red clover at concentrations of 1, fantio5, and 50 µg/mL. However, no anticoagulant activity was observed (Kolodziejczyk-Czepas et al. 2016).

Hepatotoxicity of Coumarins

Coumarins at concentrations of 130–283 mg/kg/d administered orally for two years were associated with liver toxicity in rats. No toxicity was observed at concentrations below 130 mg/kg/d (Felter et al. 2006). While animal studies can be relevant to human use, the mechanisms of coumarin metabolism in rats are significantly different than that of humans. In humans, coumarin is metabolized primarily to 7-hydroxycoumarin, a non-toxic urinary metabolite. In rats, coumarin is metabolized to 3,4-epoxide, which is quickly metabolized to o-hydroxyphenylacetaldehyde (o-HPA), a demonstrated liver toxicant (Born et al. 2000a, 2000b; Felter et al. 2006). Humans have a low rate of epoxide formation and a highly efficient rate of o-HPA detoxification. Interestingly, though mice form o-HPA at a rate even higher than that of the rat, they also clear o-HPA at a much higher rate (4-fold) than the rat, showing relative resistance to liver toxicity. This suggests that o-HPA formation alone is not solely predictive of hepatotoxicity, and that detoxification of o-HPA is a critical factor in determining toxicity. The profound differences in coumarin toxicity that exist between species, indicate that for humans the low-level coumarin exposure expected from dietary and/or supplemental sources is highly unlikely to result in toxic liver effects (Born et al. 2000a).

Despite the lack of observed hepatotoxic effects, European food safety authorities have set a maximum limit of 2 mg/kg for foods and beverages in general, and a maximum level of 10 mg/L for alcoholic beverages. The Scientific Panel on Food Additives, Flavourings, Processing Aids, and Materials in Contact with Food (AFC) (in Germany) has established a tolerable intake of 0.1 mg/kg/d body weight (= 7 mg of pure coumarin in a 70 kg adult) (Felter et al. 2006). However, there is little scientific rational for these levels (Sproll et al. 2008). As noted, a 400 mg dose of a standard red clover extract yields approximately 0.28 mg/d of total coumarins, which is magnitudes lower than the established no observed effect level of 130 mg/kg/d. No adverse liver effects associated with red clover use has been reported in humans. A review of the data for potential liver effects due to coumarin in humans suggests that any effects would be limited to ingestion of clinical oral doses of pharmaceutical coumarin (50–7,000 mg/d, for weeks or months), individuals with pre-existing liver disease, and a small subpopulation of humans lacking the 7-hydroxycoumarin detoxification pathway (Felter et al. 2006).

Lactation

Some modern clinical practitioners utilize red clover as tea for promoting lactation, usually in conjunction with other

botanicals and dietary recommendations. No safety concerns have been observed (Light 2016, personal communication to AHP, unreferenced). Formal investigations regarding the safety of red clover during lactation are lacking.

Influence on Driving

No negative effects are expected.

Overdose

No information is available.

Treatment of Overdose

No information is available.

Classification of the American Herbal Products Association

Safety Class: 1 "Herbs that can be safely consumed when used appropriately."

Interaction Class: A "Herbs for which no clinically relevant interaction are expected."

Conclusion

Red clover is a very safe botanical when used within its normal dosage range and has a high degree of safety, even at high doses. Concerns of potential bleeding due to the presence of coumarins have been reported. Concerns have also been raised regarding the potential hepatotoxicity of coumarins. These concerns are not of relevance to supplemental or therapeutic use of red clover as the concentration of coumarin in the herb and commercial extracts is magnitudes lower than the threshold required for toxicity. Additionally, plant-based coumarins are largely inactive in humans, no bleeding events have been observed clinically, and formal investigation in this area has revealed no changes in INR and prothrombin values. While there is some animal data suggesting a negative interaction with tamoxifen, all human data and other animal data supports enhanced efficacy of tamoxifen with concomitant use.

No negative clinically relevant drug interactions have been reported, nor have negative effects due to red clover's putative estrogenic activity.

INTERNATIONAL STATUS

United States

Dietary Supplement: Red clover preparations can be labeled and marketed as dietary supplement products (USC 1994), requiring FDA notification and substantiation to support permissible nutrient content and/or structure/function claim statements (FDA 2000). **Quality:** Quality standards monographs are provided in the Dietary Supplements section of the United States Pharmacopeia (USP 36) for Red Clover (the dried inflorescence containing NLT 0.5% of isoflavones, calculated on the dried basis as the sum of daidzein, genistein, formononetin, and biochanin A), Powdered Red Clover, Powdered Red Clover Extract, and Red Clover Tablets (USP 2013).

Food: Clover (*Trifolium* spp.) is classified as a spice or other natural seasoning and flavoring that is generally recognized as safe (GRAS) (FDA 2012a). Solid extract of red clover tops (*Trifolium pratense*), clover extract, clover herb distillate, and clover oil are listed as GRAS under the heading of "Essential oils, oleoresins (solvent-free), and natural extractives (including distillates)" (FDA 2011; FDA 2012b).

Australia

Trifolium pratense is a substance that may be used as an active ingredient or as an excipient of 'Listed' medicines in the Australian Register of Therapeutic Goods (ARTG) for supply in Australia. **Quality:** Where an active ingredient is covered by a monograph in the BP, PhEur, or USP then this is the minimum standard that must be applied in its entirety, otherwise a justification is required (TGA 2011a). **Indications** *Powdered red clover flower*: Traditionally used for the temporary relief of dry, flaky skin conditions (TGA 2004). *Standardized red clover extract (40 mg isoflavones)*: (1) For the symptomatic relief of medically diagnosed benign prostatic hypertrophy (TGA 2011b); (2) Aids, assists or helps in the maintenance of general well-being; (3) May assist in the management of menopause; (4) Relief of hot flushes associated with menopause; (5) Relief of menopausal symptoms (TGA 2012).

Canada

Natural Health Product (medicinal ingredient): *Trifolium pratense* plant material (fresh or dried) is classified as a medicinal Natural Health Product (NHP) under Schedule 1 of the NHP regulations, requiring pre-marketing authorization and issuance of product license for over-the-counter (OTC) human use (NHPD 2013a). **Quality:** The finished product must comply with the minimum specifications outlined in the current NHPD *Compendium of Monographs* (NHPD 2007). If a monograph is published in one of the NHPD accepted pharmacopoeias, the pharmacopoeial monograph specifications should be considered as minimum specifications used for testing of the medicinal ingredient and finished product. Pharmacopoeias and international standards currently considered acceptable by NHPD are *United States Pharmacopeia* (USP), *British Pharmacopoeia* (BP), *European Pharmacopoeia* (PhEur), *Pharmacopée Française* (PhF), *Pharmacopoeia Internationalis* (PhI), *Japanese Pharmacopoeia* (JP), *and Food Chemicals Codex* (FCC) (NHPD 2012a). **Indications** *Tincture* (1:3): Traditionally used in Western Herbalism as an expectorant in coughs and bronchial conditions. "Traditionally used in Western Herbalism as an alterative" (NHPD 2008). *Standardized extract (40.0 mg aglycone isoflavone equivalent (AIE) isoflavones (genistein, daidzein, formononetin, biochanin A)*: (1) May reduce severe and frequent hot flashes/flushes in post-menopausal women; (2) Helps to reduce bone mineral density losses with adequate intake of calcium and vitamin D in post-menopausal women; (3) May support cardiovascu-

lar health by improving arterial compliance (NHPD 2010). *Red Clover Isoflavones (40.0% isoflavones 20.16 mg AIE):* Improves symptoms associated with menopause; attenuates lumbar bone mineral density loss with adequate intake of Calcium and Vitamin D in post-menopausal women (NHPD 2012b).

Natural Health Product (non-medicinal ingredient): Certain forms of red clover are permitted for specified uses as non-medicinal components of NHPs so long as they occur at sub-therapeutic levels. The defined substance "Red Clover Flower Powder" is permitted for use as a flavor enhancer component of oral ingestion NHPs (NHPD 2013b).

European Community

Cosmetic Product: The defined ingredient "Trifolium Pratense Extract" (extract of the whole plant) is permitted for use in cosmetic products for hair conditioning and/or skin conditioning function, "Trifolium Pratense Flower" and "Trifolium Pratense Flower/Leaf/Stem Juice" for skin-conditioning function, "Trifolium Pratense Flower Extract" for astringent and/or masking function, "Trifolium Pratense Leaf Extract" and "Trifolium Pratense Symbiosome Extract" (extract of the symbiosome, the root nodule) for antioxidant and/or skin-conditioning function, and "Trifolium Pratense Seed Extract" for antioxidant function (ECHCD 2013).

Food Supplement Product: A Scientific Cooperation Project (ESCO) working group on isoflavones was set up in 2009 under the European Food Safety Authority (EFSA) with a mandate to assess the hazards for human health and the possible benefits of isoflavones, foods rich in isoflavones, including soya infant formula, as well as isoflavone supplements originating from soy or red clover (EFSA 2013, 2015). EFSA contracted with researchers at Pallas Health Research and Consultancy (Rotterdam, the Netherlands) to identify scientific data up until December 2013 on "effects of isolated isoflavones from food supplements on reproductive organs (breast, uterus) and thyroid of menopausal women" (Tijhuis et al. 2015). In 2013, the German Federal Institute for Risk Assessment (BfR) requested that EFSA provide a scientific opinion on the matter. The EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS), in October 2015, published its comprehensive review concluding that while "it was not possible to derive a single health-based guidance value for the different preparations in post-menopausal women", there was also no indication that isoflavones at levels typically found in supplements cause harm to post-menopausal women (EFSA ANS Panel 2015). Red clover isoflavone products are being marketed in some European Union member states as food supplement products.

Herbal Medicinal Product: Red clover is regulated as an active ingredient of Traditional Herbal Medicinal Products (THMPs) requiring pre-marketing authorization and product registration (EPCEU 2004). As of 2013, there are no known red clover mono-preparations with marketing authorization for use in the European Union. There is a registered THMP poly-preparation containing liquid extract (1:1) of red clover flower as one of the active ingredients in combination with liquid extracts of burdock root, sarsaparilla root, queen's delight root, cascara bark, poke root, and prickly ash bark (MHRA 2013). **Quality:** For red clover to be used as an active ingredient of a registered THMP, compliance with pharmacopoeial quality standards would be required and manufacture under pharmaceutical GMPs. Presently there are no European pharmacopoeial standards for red clover. **Indications:** Product-specific, depending on the evidence submitted by the applicant for THMP registration. As of 2013, the European Medicines Agency (EMA) has not yet prioritized the development of a Community Herbal Monograph for red clover.

World Health Organization

A monograph for Flos Trifolii, the dried inflorescences of *Trifolium pratense*, is published in the *WHO Monographs on Selected Medicinal Plants* Volume 4. ***Medicinal uses supported by clinical data: None. Uses described in pharmacopoeias and well established documents:*** Although numerous clinical trials have assessed the safety and efficacy of red clover extracts for the treatment of menopausal symptoms, hyperlipidemia, osteoporosis, and prostate cancer, the data are as yet insufficient to support any of these indications. Further data from well-controlled clinical trials with sufficient numbers of subjects are needed before any therapeutic indications can be made. ***Uses described in traditional medicine:*** Topical treatment of dermatological disorders such as psoriasis and eczema, as well as orally for the treatment of asthma and cough (WHO 2009).

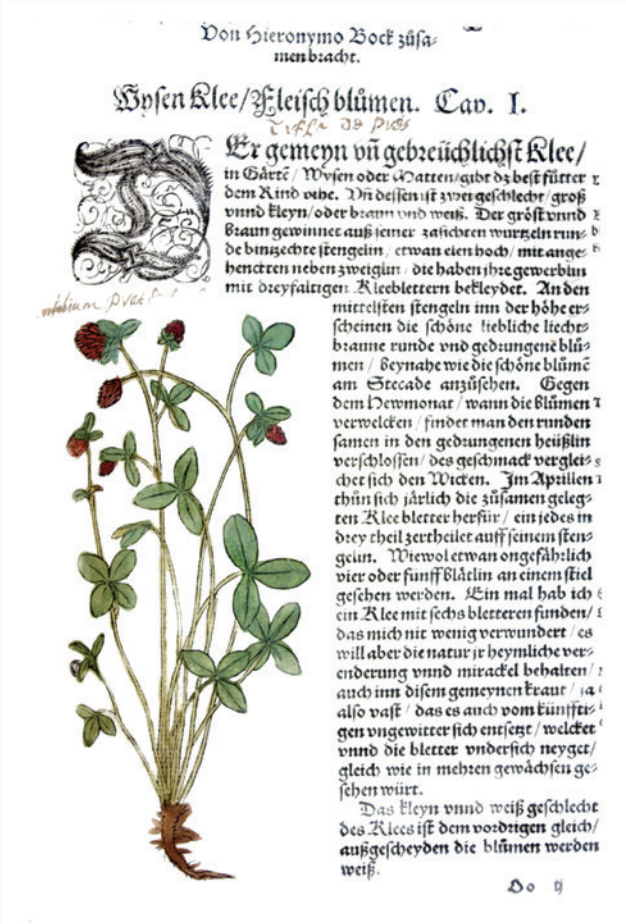
Medical Use of Red Clover in European Herbals

There is a limited amount of literature on the medicinal use of red clover, with many authorities apparently repeating uses attributed to the herb by earlier writers and few expressing personal experience with it. The writings of the Eclectics, gives us the most detailed record of the use of the herb. A number of writers ascribe the same uses (virtues) to various clovers (e.g., white, yellow, and red)

In the earliest accounts of the use of red clover in European herbals, Hieronymus Bock (1539) and Leonhardt Fuchs (1542) wrote that the flowers and seeds boiled in water and oil bring hard boils and growths to a head. This is perhaps the earliest record of using red clover for tumorous growths. A German writer, Jakob Theodor (1520-1590) also known as *Tabernaemontanus* in his *Neuwe Kreuterbuch* (1577) records Fuchs as classifying red clover as astringent and therefore of a dry nature. However, this characterization differs from later writers and modern herbalists who consider it specific for dry coughs. The same author states that Fuchs considered red clover blossoms for leucorrhea in women, the blossoms being eaten or drunk, while the white clover was said to bring on the menses.

William Turner (1568) in his *A New Herbal* recommended red clover leaves with and without flowers for the treatment of pleurisy, epilepsy, fevers, and poisonous bites, as well for its diuretic properties. John Gerard in his *The Herball* (1597), like other early references, refers to red clover as meadow trefoil. Gerard describes the leaves and flowers as being cold and dry. He explains that a decoction made with honey and used as a clyster (rectal injection) was good for "frettings and paines of the guts", driving out slimy humours that adhere there. Topically, Gerard recommended a poultice prepared by boiling the leaves in barrowes grease (pig's fat) and applied for the relief of hot swellings and inflammations. Gerard also writes of the topical preparation of clover, especially that "with the blacke halfe moon on the leafe", stamped with a little honey, for relieving pain, inflammation, and "web" of the eyes. For this purpose, the honey-clover preparation was dropped directly into the eyes.

Rembert Dodoens in his *A New Herbal* (1619) described red cover aerial parts as cold and dry, noting this can be discerned from tasting it. Boiled in mead, honied water, or wine, or as a decoction, and drunk, Dodoens reported it allays bowel inflammation. Dodoens also described the external use of red clover as a hot plaster (mixed with oil) for reducing inflamed swellings and tumors, and for cleansing wounds. John Parkinson, in his *Theatrum Botanicum* (1640), provides his first hand experience using red clover, as well as recounts the use by others (e.g. Dodoens 1619). Parkinson reports that historically, some considered red clover to be cooling and binding, while others thought it to possess a "a digesting and suppurating quality". In addition to earlier indications reported, Parkinson further writes of the folk use of the "juice" internally when bitten by an adder (snake). This may be the origin of the use of red clover for its


Von Hieronymo Bock zůſa-
men bracht.

Wyſen Klee/Fleiſch blůmen. Cap. I.

Er gemeyn vñ gebreüchlichſt Klee/
in Gärtē/ Wyſen oder Aeckern/gibt ds beſt fütter
dem Rind vihe. Vñ deſſen iſt zwey geſchlecht/ groß
vnnd kleyn/oder braun vnd weiß. Der gröſt vnnd
braun gewinnet auß ſeiner zaſichten wurtzeln rūn-
de hinzechte ſtengelin/ etwan eien hoch/ mit ange-
henckten neben zweigūn/ die haben jhre gewerbūn
mit dreyfaltigen Kleeblettern bekleydet. An den
mittelſten ſtengeln inn der höhe er-
ſcheinen die ſchöne liebliche liecht-
braune runde vnd gedrungene blů-
men/ beynahe wie die ſchöne blůmē
am Stecade anzůſehen. Gegen
dem Hewmonat/ wann die blůmen
verwelcken/ findet man den runden
ſamen in den gedrungenen heüßlin
verſchloſſen/ des geſchmack vergli-
chet ſich den Wicken. Im Aprillen
thůn ſich järlich die zůſamen gelegt-
ten Klee bletter herfür/ ein jedes in
drey theil zertheilet auff ſeinem ſten-
gelin. Wiewol etwan ongefährlich
vier oder fünff blätlin an einem ſtiel
geſehen werden. Ein mal hab ich
ein Klee mit ſechs bletteren funden/
das mich nit wenig verwundert/ es
will aber die natur jr heymliche ver-
enderung vnnd miracfel behalten/
auch inn diſem gemeynen kraut/
alſo vaſt/ das es auch vom künfftī-
gen vngewitter ſich entſetzt/ welcker
vnnd die bletter vnderſich neyget/
gleich wie in mehren gewächſen ge-
ſehen würt.

Das kleyn vnnd weiß geſchlecht
des Klees iſt dem vordrigen gleich/
außgeſcheyden die blůmen werden
weiß.

Figure 11 Historical illustration of red clover
Source: *Kreutterbuch deß Hochgelehrten unnd weitberühmten Herrn D. Petri Andreæ Matthioli* (1626) by Joachim Camerarius the Younger (1534–1598)

putative alterative properties and later reports of the herb's use for bites of venomous creatures. For this purpose, the herb was first boiled and the decoction was used to wash the bite. After washing, either a fresh poultice of bruised plant or an ointment prepared with swine grease, was applied hot directly to the bite. The topical treatment was also considered good for wounds in general and to take away scars. The decoction of the whole plant, including roots and seeds, was considered efficacious in treating leucorrhea (the whites), and a poultice of flowers and seeds mixed with oil was used to treat hard swellings and "impostumes" (abscesses).

The renowned botanist and physician Pietro Andrea Mattioli (1501–1677) repeated the aforementioned indication and further described that use of the "purplish-brown clover", that when drunk, would cause irregular menses, whereas the white clover makes the menses flow (Mattioli 1626). Nicholas Culpeper, in his *Complete Herbal* (1653) mostly restates Parkinson's and Hieronymus Bock's uses for "meadow trefoil" as an enema for stomach pains and as a poultice or ointment for inflammation, wounds, and venomous bites. William Salmon in his *Pharmacopoeia*

Londinensis (1696) and *Botanologia* (1710) gives among the most detailed description of the medical uses of red clover (whole plant) that occurs in the middle to late renaissance herbals. Salmon reports that red clover is temperate in regards to heat and cold and dry in the first degree. He ascribes "aperitive, digestive, anodyne, vulnerary, diuretic, and Alexipharmick" (anti-poisonous) properties to the plant. Indications provided by Salmon include clearing eyesight, abatement of inflammations, easing of pain, healing wounds, relieve stranguary, stopping of whites in women, resistance of poisons of venomous creatures, and curing of all fluxes (bloody discharges). Unlike many herbals of the period, Salmon gives detailed prescribing and preparations information. For most of the aforementioned uses, Salmon primarily recommended the juice or the "essence" (fresh plant mashed and blended in water) given in doses of five to six tsp. A decoction, taken in wine or water at a dose of a half pint or more morning, noon, and night, was considered useful, although weaker, for the same purposes. Topically, the juice or essence was used to allay the inflammation and pain of wounds. The seed powder was considered drying and binding and wonderfully stops the "running of the reins" (diuresis). For this purpose, two drams (approximately 3.5 grams) were given morning and night. Seed powder was also given for the cleansing and healing of foul ulcers, ruptures, and spitting of blood. An oil prepared by decocting the entire plant, or a hot poultice, applied topically was considered efficacious for contusions, venomous bites (said to draw out the poison), and inflammations, and was used to ease pain and dissolve tumors. Lastly, Salmon reported that the ashed blossoms applied topically relieved the pain and bleeding of hemorrhoids and treated falling anus.

Lonicero (1679) added a bit to the historic uses of clover, stating that three leaves of the clover taken drives away malarial fever. Tournefort, in his *Compleat Herbal* (1719), says it is not used in physic (by physicians) but that the earlier German botanist/physician Tragus (Hieronymus or Jerome Bock), in his *New Kreuterbuch* (1539), recommended "the flowers and seeds boiled in wine to ease labor pains and to cut the viscid substances which are found in the intestines.". Bock noted "clover flower when boiled in honey water or wine drunk or given as an enema soften the tough hard phlegm in the bowels, healeth any injury and eases the pain", working in a fashion that was similar to fenugreek seed (*Trigonella foenum-graecum*). Bock also recommended the topical application of the flowers and seeds noting that boiling with water and oil "bring hard lumps and ulcers to a boil" and considered white clover to have the same properties. Tournefort also mentions the flowers and seeds can be "boiled in water or oil and applied as a cataplasm to discuss tumors, where there is no inflammation".

Use of Red Clover in Asia, the Middle East, and the Caucasus

A variety of *Trifolium* species have been used by Oriental and European cultures for the treatment of eczema and psoriasis. In China, red clover (*san xiao cao*) is classified as neutral and somewhat sweet and is used in a similar manner as in the west, namely to clear heat and cool the blood (actions that make it specific for skin conditions and infections), and additionally is used to treat withdrawal (Garran 2008), though it does not seem to be a prominent therapeutic agent in this tradition. In Turkish traditional medicine, *T. pratense* as well as *T. repens* and *T. arvense* have been used for their expectorant, antiseptic, analgesic, sedative and tonic effects (Sabudak and Guler 2009). *T. pratense* and *T. repens* are reportedly popular in Pakistan in the treatment of sore throat, fever, pneumonia, meningitis, and feverish feeling (Khan and Khatoon 2008). In the Albanian Alps in Kosovo, a juice obtained from squeezed leaves of *T. pratense* is a folk medicine for stomach disorders while *T. repens* decoctions were applied for the treatment of diarrhea (Mustafa et al. 2012).

The infusion of red clover aerial parts were used by the people of the Caucasus for the treatment of gynecological diseases. A tincture prepared from the flowers is used to treat gout and poisonings. Topically, flowers are used for their wound-healing properties, for infections of the fingernails (paronychia), and for ear and eye diseases (Sokolov 1985–1993 in Mamedov et al. 2015).

Medical Use of Red Clover in the Americas

The most noted medical uses of red clover among Native Americans were for whooping cough, as a "blood medicine", leucorrhea, and for cancer, most specifically as an infusion and decoction (Herrick 1995; Moerman 2013). There was one report of the historical use of red clover by the Iroquois for "change of life" (Herrick 1995) suggesting some use for menopause. No specific dosing information regarding these uses is available.

Samuel Thomson (1822) reintroduced red clover into Western herbal medicine use and utilized red clover blossoms as part of a widely used "cancer plaster". The plaster was made by boiling red clover flowers in water for one hour in a brass kettle. The spent flowers were then replaced with fresh flowers, and the new flowers also boiled for an hour. The resulting decoction was strained out and the mixture was carefully reduced until it had the consistency of tar. The salve was used for "cancers, sore lips, and all old sores". One of Thomson's disciples, AI Coffin took his unique system of medicine to England where it flourished and created the foundation of modern British phytotherapy. Coffin, in his major text, *The Botanic Guide to Health* (1852) repeats Thomson's use of the salve for topical lesions and discusses its very successful use in a case of longstanding cancer. Back in the US Thomson's rival, Wooster Beach the founder of Eclectic medicine, also recounts Thomson's use of his "cancer plaster", stating it has "emollient and antiseptic" effects, making it "very useful for old sores, scrofulous tumors, sore lips, etc. (Beach 1852). However, Beach discounts the salve's benefits for actually treating cancer, saying "its reputation as a cancer-plaster is far from being a deserved one".

Additional uses for red clover are recorded in the *Indian Household Medicine Guide*, written by "The Great Indian Medicine Man", JI Lighthall (1883). Lighthall primarily recommended the blossoms as a tea for "irritation of the

vocal cords, windpipe, and bronchial tubes". He goes on to report that the tea is beneficial for asthma, cold, coughs, and hoarseness. These uses are quite similar to earlier English uses. Lighthall's favorite formula for using red clover was to make a syrup from the tea mixed with honey and the juice of roasted onions (*Cepa* spp.). This, he says, makes an excellent cough syrup for a wide array of upper respiratory problems. The same indications persisted in the eclectic literature, which recorded irritation of the bronchial, laryngeal, and pulmonary surfaces with spasmodic cough. Secondarily it was considered of use in measles, pthisis, and general pulmonary complaints, as well as being a specific for pertussis, and a valuable alterative (Cooper and Bloyer 1892; Ellingwood and Lloyd 1919).

Eclectic and Physio-Medicalist Use of Red Clover

Reports of the use of red clover among early American physicians in the middle 1800s seems to have been sporadic, reflecting a relative lack of experience regarding the herb's efficacy. This is evidenced by numerous writers recording that it was equally ineffective as it was effective, and referring to the writings of earlier authors rather than expressing their own experience. This changed over time as in 1917, pharmacognosist Lucius Sayre reported on the quite extensive use of the flowering tops as an alterative and as deobstruent and sedative for whooping cough, reporting on the use of white clover infusion for whooping cough as well.

The physio-medicalist and Eclectic physicians were responsible for promoting a wider understanding of this common plant's uses. The physio-medicalists were the licensed medical descendants of the earlier Thomsonian/Botanic "doctors". William Cook, in his P*hysio-Medical Dispensatory* (1869) reported that the herb was not much used in medicine, but that a decoction prepared from the plant (parts not specified) is "somewhat antispasmodic and "enjoyed a good reputation" for hooping cough. Cook further records that an extract made by evaporating the decoction is a stimulant (not otherwise specified) and was valuable in indolent ulcers and cancer. Interestingly, Cook noted that red clover had a good reputation for treating cancers and that, while the herb was not escharotic, it "is too sharp to be applied alone", was best made into an ointment or plaster with milder extracts, and that it "secures a good discharge, arouses a firm capillary circulation, and procures a granulating surface to indolent and phagedaemic sores". The prominent physio-medical physician, TJ Lyle (1897), notes, "the blossoms are a mild stimulating and relaxing alterant" (alterative). He reiterates that plaster has a reputation for treating topical cancers, as well as scaly skin and indolent ulcers, and gives a formula for a compound syrup of *Trifolium* containing red clover blossoms, poke root (*Phytolacca americana*), cascara amarga, potassium iodide, and prickly ash bark (*Xanthoxylum americana*), which was to be used internally for syphilis and "suppurating glands". This same formula is almost identical to the Trifolium Compound made by Parke Davis Co, the William Merrill Chemical Co's Extract Trifolium Compound, and the secret formula of the unlicensed "cancer doctor" Harry Hoxsey (the Hoxsey formula). Lyle also noted the hot infusion of red clover blossoms improves capillary circulation, has a mild laxative effect, and soothes the nerves. Lyle recommended combining the fluidextract of red clover with a vinegar extract of lobelia (*Lobelia inflata*) for treating whooping cough (pertussis), but unfortunately provided no clear dosing information.

The prominent Eclectic physician, Harvey Wickes Felter essentially agrees with Lyle's use of red clover, recording the herb as being especially useful for the dry, irritated, and spasmodic coughs of pertussis and measles and that it also moderates the cough of bronchitis and laryngitis. In coughs of pertussis and in measles, Felter carefully notes that this remedy "fails as often as it succeeds", but it is worth trying. Conversely, Felter says that red clover is underrated as an alterative and should be utilized for treating chronic skin diseases (orally) and that it "unquestionably has a retarding effect upon malignant neoplasms" (Felter 1922). Felter (1922) recommends a dose of one to 10 drops of specific *Trifolium* (Lloyd Brothers) every two to three hours. While Felter states the herb is not curative, he says post-surgical cancer patients have better outcomes and that regrowth of the tumor is slowed when the tincture of red clover is taken daily. Regarding the use of red clover in bronchial conditions, Scudder (1903) levied the same caution in his earlier writings that red clover is not always effective, but when it is, it is "speedy and permanent."

The fluidextract of red clover blossoms and a compound syrup of *Trifolium* were included in the *National Formulary* (NF) beginning in 1926. A number of other representative formulas were provided by various herbal authorities of the time (see Red Clover Formulas). In the 20th century, the noted German naturopathic physician, Otto Mausert in his *Herbs for Health* (1932) described red clover as a depurative and pectoral describing red clover as an effective blood purifier; anise is an aromatic tonic; damiana invigorates the body and nerves; sa tea (strawberry leaf) is an effective alkalizer; woodruff aids digestion and quiets the nerves. Drunk as an ordinary tea (infusion) as a general relaxing nervine for general nervousness.

About the same time, the neo-Thomsonian Jethro Kloss, based on his reported personal experience using red clover clinically, considered red clover to have depurative (blood purifying), detergent, alterative, and mild stimulant effects. Kloss stated that "Red Clover is one of God's greatest blessings to man" (Kloss 1939), extolling its virtues as a blood purifier. In this regard Kloss suggested the combination of red clover with equal parts of blue violet leaf (*Viola* spp.), burdock seed (*Arctium lappa*), yellow dock root (*Rumex crispus*), dandelion root (*Taraxacum officinale*), rock rose (*Cistus albidus*), and goldenseal root (*Hydrastis canadensis*) to treat cancerous growths, leprosy affections, and pellagra. Kloss, a 7th Day Adventist, also quotes from Ellen G White, the founder of the 7th Day Adventist Church, on the use of this humble plant. According to Kloss and White, it is used for quieting the nerves, for relieving spasmodic coughs, and topically as a salve for wounds and difficult to heal sores, a unique use of red clover that is consistent with the ethnobo-

tanical use of the plant in Turkey (Renda et al. 2013). Kloss also gives a formula he considered "splendid for syphilis" and "exceedingly good for cancer in any part of the body", especially esophageal and gastric cancers (see Red Clover Formulas)

In throat cancer, Kloss instructed to make a strong tea for a gargle four to five times daily, swallowing some of the tea. In stomach cancer, Kloss recommended to that four or more cups daily be drunken on an empty stomach. For external sores, red clover was to be used freely in a bath. In rectal cancer, the tea was to be injected with a syringe five to six times daily. In uterine cancer, a douche was to be used and retained for several minutes. Used singularly, Kloss also considered red clover "excellent" for stomach cancer, whooping cough, and various spasm (Kloss 1939).

Red clover appears in the early North American herbal materia medica. In their course work, the Dominion Herbal College (1926) included red clover among their featured alteratives, recommending a warm infusion for spasmodic coughs, whooping cough, and to soothe the nerves and a red clover formula from a Dr England for the treatment of syphilis (see Red Clover Formulas). The Dominion Herbal College (1926) course work also reports on the reputation of red clover as a wash for scaly skin, noting the extract was highly recommended for cancer and indolent ulcers and that it promotes healthy granulation. In commenting on the red clover cancer plaster of Samuel Thomson, it notes that application of the plaster causes many patients to complain of "severe smarting" and recommend that it be mixed with an extract of dandelion to mollify the irritation.

Kuts-Cheraux (1953) in his *Naturae Medicina and Naturopathic Dispensatory* reported antispasmodic, sedative, antitussive, and alterative properties for red clover blossoms. Kuts-Cheraux included paroxysmal coughs due to bronchial or laryngeal irritation as a primary indication and recommends it combined with sundew (*Drosera* spp.) or elecampane root (*Inula helenium*). This writer also reports that red clover possesses mild but distinct alterative properties used for many debilitating disorders and mild cachexias.

John Christopher, who was in many ways the godfather of the modern American herbal renaissance, also used a red clover formula that was an obvious modification of the earlier *Trifolium* Compounds consisting of red clover blossoms, chapparal, licorice root (*Glycyrrhiza glabra*), poke root (*Phytolacca Americana*), peach bark (*Prunus persica*), Oregon grape root, stillingia root (*Stillingia sylvatica*), prickly ash bark, burdock root, and buckthorn bark (*Frangula* spp.) for purifying the bloodstream and treating cancer, syphilis, scrofula, and other wasting diseases (Christopher 1976). Christopher also recommended that a strong tea of the herb be used as an enema for rectal cancer and a retention douche for cervical/vaginal cancers.

Priest and Priest (1982) in their *Herbal Medication* clinical and dispensary handbook (UK), record red clover as a mild stimulating and relaxing alterative with special affinity for throat and salivary glands. These authors especially consider red clover indicated for debilitated children with chronic bronchial or throat conditions. Secondarily, they attribute antispasmodic, sedative, and expectorant actions to the herb, recommending fluidextract doses of up to 4 mL/d.

Modern day herbal clinicians still use red clover for many (but not all) of its traditional uses. It is unlikely anyone considers red clover of significant benefit for pellagra, syphilis, or scrophula (tuberculosis of the parotid glands). Yet the herb's use persists. The late naturopathic physician/herbalist William (Bill) Mitchell used it regularly in his clinical practice combining red clover blossoms with Oregon grape root for eczematous skin problems; with alteratives such as burdock root, blue flag (*Iris versicolor*), pipsissewa (*Chimaphila umbellata*), and wahoo bark (*Eunonymus atropurpureus*) for lymphatic cancers; and with licorice root (*Glycyrrhiza* spp.) and grindelia buds (*Grindelia* spp.) for whooping cough. Mitchell also mentions that his mentor, Dr John Bastyr, used red clover to treat mild cachexia as well as upper respiratory tract irritation and coughs (Mitchell 2003).

Iconic American herbalist, Michael Moore, believed the herb's reputation for treating cancer, autoimmune disease (rheumatoid arthritis; not a historical use), and for menopausal symptoms are gross exaggerations (Moore 2003). Moore did regard red clover as a good tasting, mineral rich tea with mild alterative, expectorant, antitussive, and sedative effects, and that its "value as a medicine lies in its mildness and good taste, making it one of the best maintenance liquids for the duration of any infection". Moore specifically mentions its use as a tonic in recovering from debilitating illnesses such as hepatitis and mononucleosis (Moore 2003).

Other modern herbalists (e.g., Hoffmann 1983; Mills and Bone 2000; Romm 2010) report a variety of new indications, partially based on the use of an isoflavone-rich extract (Promensil) used for menopause. Among these indications are included: hot flashes, vaginal dryness, prevention and treatment of osteoporosis, hypercholesterolemia, and as a substitute for hormone replacement therapy (Romm 2010); as a lymphatic and expectorant and for skin and joint diseases (Mills and Bone 2000); and for psoriasis (combined with yellow dock root [*Rumex crispus*] and stinging nettle [*Urtica dioica*]) (Hoffmann 1983). British herbalist Anne McIntyre (1996) additionally considers red clover a specific for children with a complex of eczema and asthma and recommends fomentations made from the infused blossoms as a douche for vaginal infections, topically for inflamed nipples, as a gargle for sore throat, and as an eyewash.

Red Clover Formulas

Fluid Extract of Trifolium (National Formulary 1926)

Red clover (moderately coarse powder)	**1,000 g**

Prepare by percolation using diluted alcohol (one volume of alcohol and three volumes of water; 36% to 40% alcohol) as the menstruum and reserving the first 800 mL of the percolate. Moisten the ground herb with a sufficient quantity of menstruum to render it evenly and distinctly damp, and let stand for six hours in a tightly covered container. Pack in percolator then add enough menstruum to saturate the powder leaving a stratum of menstruum above the powder. When the liquid begins to drop from the percolator, close the lower orifice and allow to macerate for 48 hours. Allow to percolate slowly gradually adding more menstruum until the material is exhausted. Reserve the first 800 mL. Recover the alcohol from the remainder and concentrate the residue to a soft extract at a temperature not exceeding 60 ˚C. Dissolve this in the previously reserved portion, mix thoroughly, and add a sufficient quantity of the menstruum to obtain 1,000 mL.

Dose	4 mL

Compound Fluidextract of Trifolium (National Formulary 1926)

Red clover *Trifolium repens*	215 g
Licorice *Glycyrrhiza glabra*	215 g
Barberry *Berberis spp.*	108 g
Cascara amarga *Picramnia antidesma*	108 g
Burdock root *Arctium lappa*	108 g
Poke root *Phytolacca americana*	108 g
Stillingia *Stillingia sylvatica*	108 g
Prickly ash bark *Xanthoxylum americanum*	30 g
Percolate as described for Trifolium Fluid Extract above.	
Dose: 4 mL	

Compound Syrup of Trifolium (National Formulary 1926)

Fluidextract of Trifolium	300 mL
Powdered tragacanth	1 g
Oil of sassafras	0.4 mL
Oil of anise	0.2 mL
Methyl salicylate	0.2 mL
Sucrose	650 g
Distilled water	QS to 1,000 mL

Mix tragacanth with fluidextract, add oils and methyl salicylate and 250 mL of distilled water; add sucrose and agitate until dissolved. Add sufficient quantity of water to make 1,000 mL and mix thoroughly.

Dose 8 mL

Herb Health Tea (Mausert 1932; Formula 225)

Red clover	3 ounces
Anise seed *Pimpinella anisum*	2 ozs
Damiana leaves *Turnera diffusa*	2 ozs
Sa tea *Fragaria vesca*	8 ozs
Woodruff herb *Galium odoratum*	1 oz
Dose: One tsp of herb per cup of boiled water.	

Red clover alterative formula for syphilis and cancer (Kloss 1939)

Red clover	1 oz
Burdock seed	1 oz
Oregon grape root *Mahonia aquifolium*	2 oz
Blood root *Sanguinaria canadense*	1/2 oz
Infuse in one pint each of hot water and hot apple cider.	
Dose: One wineglassful four times daily.	

Red Clover Alterative and Bronchial Tea (Dominion Herbal College 1926)

Red clover blossoms	1 oz
Burdock seed	1 oz
Mullein leaf *Verbascum thapsus*	½ oz
Yellow parilla root *Menispermum canadense*	1 oz
Simmer in two qts water down to one qt.	
Dose: One wineglassful three to four times daily.	

Red clover tincture for syphilis (Dominion Herbal College 1926)

Oregon grape	7.3 g
Red clover	4.7 g
Burdock seed	4.4 g
Cascara sagrada *Frangula purshiana*	3.8 g
Blue flag *Iris versicolor*	3.4 g
Prickly ash berries	2.3 g
Blood root	2.3 g
Macerate in 50% alcohol to make 568 mL (1:20 w/v) of tincture.	
Dose: One tsp four times daily, sometimes for up to one year.	

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TRIFOLIO DE PRATI.

peroche vitrono hauerne fatto egli mentione nel quarto libro, descriuendo il loto saluatico, con queste parole. Il Loto saluatico nasce copiosissimo in Libia, con fusto alto due gombiti, & spesso maggiore, con molte concauità d'ali. & con frondi simili al trifoglio de i prati. Dalle quali parole si conosce quanto erri il Gesnero nel suo libro de gli animali, uolendo egli che il Trifoglio de prati sia il Loto. Di tre spetie di Trifogli scrisse Plinio al IX. cap. del XXI. libro, cosi dicendo. Il Trifoglio è di tre sorti. i Greci lo chiamano menianthes, & altri asphaltion, di maggiori frondi: il quale usano coloro, che fanno le ghirlande. Il secondo produce le frondi acute, & imperò è chiamato oxitriphillon, cio è Trifoglio acuto. Il terzo è molto piu minuto di tutti questi. Scrissene una spetie d'acuto Scribonio Largo, in questo modo dicendo. Nasce il Trifoglio acuto copiosissimo in Sicilia: & non l'ho mai ueduto io in Italia, se non nel porto di Luni, quando

Trifoglio acuto scritto da Scribon.

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