Review

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Phytochemical profile and pharmacological properties of *Trifolium repens*

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Abstract: *Trifolium repens* belongs to the family Leguminosae and has been used for therapeutic purposes as traditional medicine. The plant is widely used as fodder and leafy vegetables for human uses. However, there is a lack of a detailed review of its phytochemical profile and pharmacological properties. This review presents a comprehensive overview of the phytochemical profile and biological properties of *T. repens*. The plant is used as antioxidants and cholinesterase inhibitors and for anti-inflammatory, antiseptic, analgesic, antirheumatic ache, and antimicrobial purposes. This review has summarized the available updated useful information about the different bioactive compounds such as simple phenols, phenolic acids, flavones, flavonols, isoflavones, pterocarpanes, cyanogenic glucosides, saponins, and condensed tannins present in *T. repens*. The pharmacological roles of these secondary metabolites present in *T. repens* have been presented. It has been revealed that *T. repens* contain important phytochemicals, which is the potential source of health-beneficial bioactive components for food and nutraceuticals industries.

Keywords: antioxidants; phytochemistry; secondary metabolites; *Trifolium repens*.

The *Trifolium* genus is usually found in subtropical and temperate regions of both sides of the hemispheres, and it is comprised of about 240 species [1]. It is common in the Mediterranean region, where 103 species of *Trifolium* have been reported [2]. For several decades, some species of *Trifolium* such as *Trifolium resupinatum*, *Trifolium medium*, *Trifolium pratense*, *Trifolium subterraneum*, *Trifolium pannonicum* Jacq, *Trifolium fragiferum*, *Trifolium hybridum*, *Trifolium incarnatum*, and *Trifolium repens* have been used as forage plants [3]. Besides, some species of *Trifolium* are used as important valuable herbs in folk medicine in different cultures across the world [4–6]. Currently, *Trifolium* plants are used as a therapeutic agent. This usage was based on a recommendation as traditional medicine. The scientific research on the biological activity of different species of *Trifolium* and their conceivable therapeutic effects has been growing as shown in Table 1. Several studies had reported the biological properties of *Trifolium* species, especially the red clover (*T. pratense*). It was found that it contains flavonoids, isoflavonoids, and other different antioxidant compounds [7]. Owing to the presence of these valuable compounds, some *Trifolium* species are used as therapeutic agents as antiseptic, analgesic, anti-inflammatory, and anti-cancer agents and in angiogenesis [8]. The beneficial effects of *Trifolium* may be dependent on the mechanistic action of different biologically active compounds present in these herbs.

Introduction

The clover is also known as *Trifolium* and belongs to the family Leguminosae, an important family in terms of its agricultural value and the number of species it contains.

Natural occurrence of *Trifolium repens*

Trifolium repens are also known as white clover, white trefoil, Dutch clover, creeping *Trifolium*, honeysuckle clover or ladino clover [9]. Among all species of *Trifolium* in the genus, *T. repens* is one of the highly important sources of food and feed [10]. It is believed to be originated from the Mediterranean in the early Miocene period, 16–23 million years ago [11] and was spread through Western Asia and Europe through the migration of animals as well as humans. The domestication of *T. repens* started about 400 years ago, and now, it is considered a naturalized plant [12]. *T. repens* tend to acclimatize in the temperate region of the

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globe where more than 750 mm annual rainfall occurs [13]. The distribution of *T. repens* is very wide due to its edaphic tolerance nature. It grows on soils ranging from markedly acid to calcareous [14]. Thus, more than a hundred cultivars are known to exist [15]. Generally, the places in the world in which *T. repens* grow are New Zealand, Australasia, Western Europe, North America and South Asia.

Role in modern agriculture

T. repens is used in a mixed form with other grasses in the world. Generally, it is used as grazing, pasture hay and also acts as a groundcover in horticultural condition. It also plays a significant role in the wool, dairy and meat industries in terms of highly increasing qualities of the

Table 1: Compounds present in *Trifolium repens* and their possible role in biology.

Class	Subclass	Compounds	Effect	References
Flavonoids	Flavones	5,6,7,8-Tetrahydroxy-4'-methoxyflavone	Toxic to insects, show anti-inflammatory activity, reduce high blood pressure	[28–32]
		7,4'-Dihydroxyflavone		
		Acacetin		
		4',5,6,7,8-Pentahydroxyflavone		
		Luteolin		
		Geraldone (4',7-Dihydroxy-3'-methoxyflavone)		
Flavonols	Flavonols	3',4',7-Trihydroxyflavone	Show antifungal, antibacterial, and insecticidal activity and enhance nitrogen fixation	[33–35]
		Quercetin		
		Kaempferol		
		Myricetin		
		Isorhamnetin		
		6-Hydroxykaempferol		
		4',7-Dihydroxyflavonol		
		7-Hydroxy-4'-methoxyflavonol		
Isoflavonoids	Condensed tannins	Rhamnetin	Show antimicrobial, antileishmanial, and antifungal activity	[36–39]
		Prodelphinidin		
	Isoflavones	Pratensein	Show antifungal and antibacterial activity and reduce postpartum depression and skin aging	[40–44]
		Glycitein		
		Genistein		
		Calycosin		
		Biochanin A		
		Daidzein		
		Formononetin		
		Pseudobaptigenin		
		7,2',4'-Trihydroxyisoflavone		
		2'-Hydroxyformononetin		
	Isoflavanones	Vestitone	Fungitoxic	[10, 45]
		7,2',4'-Trihydroxyisoflavanone		
	Coumestans	Coumestrol	Show antifungal, anti- <i>Helicobacter pylori</i> , anti-inflammatory, antiaging, and antihepatotoxic activity	[46–49]
		Repensol		
		Trifoliol		
	Pterocarpanes	Daphnoretin	Have antibacterial effect and antifungal properties	[46, 50]
		Medicarpin		
		Demethylmedicarpin		
Cyanogenic glucosides		Linamarin	Show antifungal activity and have antidiabetic properties	[51, 52]
		Lotaustralin		
Saponins		Astragaloside VIII	Have hepatoprotective effect and show antifungal activity, anti-inflammatory, antioxidant, and health-promoting activities	[53–56]
		Cloversaponin I–V methyl ester		
		Azukisaponin II methyl ester		
		Soyasaponin I		
		Soyasaponin I methyl ester		
		Soyasaponin II methyl ester		

products [13]. As a fodder additive, it was found to significantly improve milk quality [16]. There are several other important uses of *T. repens* in a pasture due to the presence of high nutritive values, a rich source of proteins and minerals, which ultimately provide a significant increase in the qualities of products delivered by animals [17]. Besides, recently it has been investigated that *T. repens* have the potential for different metal (cadmium and zinc) accumulation in the roots [18].

Genetically modified *T. repens* have shown different traits including Alfalfa mosaic virus resistance, white clover mosaic virus resistance, insect resistance, and nutritional quality alteration. Besides, *T. repens* were also modified for the development including nutrient efficiency, bloat safe lines, aluminum tolerance, and delayed leaf senescence. But there is no commercial approval of genetic modification in *T. repens* [19–21]. However, some potential genes have been identified to be involved in flower pigmentation and other chemical characteristics of white clover [22–24]. Hybrid varieties have been developed with enhanced physicochemical and biological potential, especially from *T. repens* and *Trifolium uniflorum* [25]. However, genotypic and phenotypic diversity does not affect productivity and drought response in competitive stands of *T. repens* [26]. There is no evidence available that insufficient pollination occurred in *T. repens* [27], making it a favorable agricultural product.

There is a lack of literature on biological compounds in *T. repens* and their medicinal evaluation. Valuation of the innovative therapeutic bioactive potential of *T. repens*-derived extracts seems to have a strong tendency in experimental phytopharmacological research in a variety of treatments. The low cost of cultivation, the variety of different bioactive potential compounds, and prevalent occurrence are advantages of *Trifolium* species. This article critically reviewed the available data on physiochemical activity and medicinal valuations of *T. repens* and also shows the future scenarios for the usage of *T. repens* as therapeutic agents in different biological disorders. The search keywords were “*Trifolium*”, “*Trifolium repens*”, “phytochemical profile” and “medicinal uses”. These keywords were searched in “Google scholar”, “Scopus” and “web of science” databases without any date restriction.

Trifolium in traditional medicine

All species of *Trifolium* have been known to act as traditional medicine [57]. Oriental and European cultures used different species of *Trifolium* for the therapy of psoriasis and eczema. In

Turkey, *T. pratense*, *Trifolium arvense*, and *T. repens* are used as traditional medicine as analgesic, antiseptic, sedative, expectorant, and tonic mixture [58]. In Iran, the aerial parts of *T. repens* are used to treat neonatal jaundice and dermal disorders [59] and leaves and inflorescence as an analgesic. In Europe, juice of *T. pratense* has been used for common stomach disorders, while the decoctions of *T. repens* have been used as a conventional antidiarrheal medication [60]. In America, *T. pratense* has been used for the treatment of lung problems, external skin problems, and nervous and reproductive disorders [61]. *T. pratense* contains isoflavone constituents, which have estrogenic properties and is used against the complication of menopausal disorders such as breast cancer, cardiac risk factors, and osteoporosis [62]. *T. pratense* (red clover) extracts are commercially available dietary supplements in the European and US market [63]. *T. repens* are used as traditional medicine as a deworming agent in some parts of the world [64]. In Naga tribes of India, *T. repens* are used as a deworming remedy because of the anticestodal activity of the aerial shoots [65]. In Pakistan, white clover (*Trifolium repens*) and red clover (*Trifolium pratense*) species are popular plants for the treatment of fever, feverish feeling, meningitis, pneumonia, and sore throat infection [66]. In the Alpine regions of Pakistan, the extract or drinks of the whole plant of *T. repens* is used to treat abdominal pain, joint disorder, coughs, colds, and fever and for eye wash [67, 68]. The traditional use of *Trifolium* species including *T. repens* indicates that it has valuable bioactive compounds which may act as a remedy in different biological disorders.

Phytochemistry of *Trifolium*

The chemistry of the *T. repens* is partly known. Ruckle et al. [69] reported glucose, fructose, sucrose, and starch contents in differently grown *T. repens*. The leaves are rich in pigments such as chlorophylls [70]. Besides, the most commonly known biochemical compounds such as isoflavones are widely present. The species synthesizes an extensive range of different phenolic and polyphenolic compounds such as saponins, flavonoids, clovamide, acids of phenol, and other substances [71]. Tava et al. [72] studied the phenolic contents of fourteen *Trifolium* species including *T. repens* as shown in Table 2. The authors showed that *T. repens* has the lowest amounts of phenolic compounds. They established a link between the total phenolic contents and environmental factors. The flowers of *T. repens* also contain several important phenolic compounds [73]. Elgersma et al. [74] showed that *T. repens* is a good source of A-tocopherol, B-carotene, lutein, protein, and fibers. Chen et al. [32] identified 169 different classes of

compounds using ultra performance liquid chromatography (UPLC-MS). These include organic acids, alkaloids, amino acids, peptides, flavonoids, oligosaccharides, and coumarins. A recent study using ultra-performance liquid chromatography-high resolution mass spectrometry (UPLC-HRMS) revealed 29 phenolic compounds in *T. repens* [75]. The highest amount was of tyrosol (11.1 mg/g), quercetin-3-glucuronide (5.36 mg/g), formononetin-7-glucoside (5.03 mg/g), quercetin-3-O-glucoside (4.71 mg/g), 3, 4-di-O-caffeoylquinic acid (4.69 mg/g), formononetin-7-glucoside-acetate (4.29 mg/g), quercetin-3-O-glucoside (4.26 mg/g), and formononetin (3.64 mg/g). The detailed phytochemistry including classes, subclasses, and compounds in the subclasses and their possible biological effects on animals and humans is given.

Flavonoids

Flavonoids such as anthocyanins are responsible for color and are a pigment which further ensures fertilization by pollination and dispersion of seeds by animals [76]. Phenolic compounds are the integral parts of both human and animal diet, which are present almost in all parts of the plants, especially in those parts of the plants, which carry out photosynthesis [77]. Flavonoids provide a key role in human nutrition which ultimately acts as health-promoting natural chemicals [76, 78, 79]. Flavonoid is present in all groups of the kingdom Plantae [80]. Each group of flavonoid compounds is responsible for playing a specific role in the plant. Flavonoids such as afzelechin, catechin, gallicocatechin,

epiafzelechin, epicatechin, and epigallocatechin, condensed tannins such as prodelphinidins and procyanidins, and their mixed oligomers were reported to be present in *T. repens* [81].

The quantitative analysis of *T. repens* reveals that it contains flavonoid subclass flavones including compounds such as acacetin, luteolin, geraldone (4',7-dihydroxy-3'-methoxyflavone), 5,6,7,8-tetrahydroxy-4'-methoxyflavone, 7, 4'-dihydroxyflavone, and 4',5,6,7,8-pentahydroxyflavone. Another study was conducted by Ponce et al. [82] for the identification and isolation of flavonoids in *T. repens* using spectrophotometric and chromatographic techniques. The plant was cultivated either in the absence or presence of mycorrhizal fungi. Flavones 5,6,7, 8-tetrahydroxy-4'-methoxyflavone, 4',5,6,7, 8-pentahydroxy-3-methoxyflavone, 3,5,6,7,8-pentahydroxy-4'-methoxyflavone, 6-hydroxykaempferol, 4',5,6,7,8-pentahydroxyflavone, 3,4'-dimethoxy kaempferol, tetrahydroxy-3-methoxyflavone, and 3,7-dihydroxy-4'-methoxyflavone were identified (Figure 1).

Hofmann et al. [83] identified flavonoids in *T. repens* against the response of the plant population to UV-B radiation. They isolated flavonoids from the methanol-water extract and quantified by high performance liquid chromatography (HPLC) indicating that major flavonols are the derivatives of quercetin and kaempferol.

The extract of *T. repens* contains three main flavonoids, that is, quercetin-3-galactoside, 6'-acetyl derivative, and myricetin-3-galactoside, which were isolated and identified by the spectroscopic method [84]. The larvae of butterfly (*Polyommatus icarus* Rott) were nurtured on *T. repens*, and the extract from the individual larvae, pupae, and larval feces

Table 2: The amounts of phenolic compounds present in leaves of 14 *Trifolium* samples [60].

<i>Trifolium</i> SP.	Phenolic group concentration (mg/g dry matter)					
	Phenolic acids	Clovamides	Isoflavones	Other flavonoids	Proanthocyanidins	*Total phenolics
<i>T. alexandrinum</i>	4.66	8.53	4.20	41.15	4.10	62.65
<i>T. pratense</i>	5.94	29.86	22.63	13.78	0.66	72.89
<i>T. pratense</i> subsp. <i>nivale</i>	4.68	8.64	16.55	16.88	0.14	46.90
<i>T. repens</i>	1.40	—	0.31	9.19	0.05	10.96
<i>T. subterraneum</i>	2.00	—	70.03	7.55	0.06	79.64
<i>T. subterraneum</i>	1.87	—	48.00	7.94	0.03	57.84
<i>T. pratense</i>	2.85	22.33	22.72	21.08	0.12	69.11
<i>T. repens</i> var. <i>giganteum</i>	1.27	—	0.70	6.18	0.25	8.42
<i>T. alpinum</i>	—	—	0.38	27.68	0.37	28.44
<i>T. badium</i>	—	—	0.17	34.70	8.58	43.45
<i>T. ochroleucum</i>	2.42	—	0.54	44.14	0.39	47.51
<i>T. pratense</i> subsp. <i>nivale</i>	1.71	3.89	14.56	28.54	0.32	49.02
<i>T. repens</i> var. <i>sylvestre</i>	—	—	0.56	14.39	0.04	14.95
<i>T. thalii</i>	1.02	—	0.79	18.50	0.47	21.79

* μ mol gallic acid equivalents/100 g freeze-dried matter.

Figure 1: Structure of the important phenolic compounds present in *Trifolium repens*.

was analyzed by HPLC. Their study suggested that *T. repens* is a good source of phenolic compounds. In conclusion, *T. repens* possess flavonoids, which play an important role in the physiological activities in both plants and animals.

Flavonols

The analysis of *T. repens* using liquid chromatography with tandem mass spectrometry (LC-MS/MS) showed to contain

different flavonols such as kaempferol in the concentration 286.24 g/mol, astragalin (448.39 g/mol), kaempferol-rhamnoside-glucoside (594.53 g/mol), kaempferol-rhamnoside-xyloside-galactoside (726.63 g/mol), quercetin (302.24 g/mol), hyperoside (464.38 g/mol), quercetin-xyloside-galactoside (596.49 g/mol), and quercetin-rhamnoside-xyloside-galactoside (742.63 g/mol). *T. repens* contain simple flavonols such as quercetin, kaempferol, myricetin, isorhamnetin, 6-hydroxykaempferol, 4',7-dihydroxyflavonol, 7-hydroxy-4'-methoxyflavonol, and rhamnetin [33–35]. Owing to the

presence of these bioactive valuable compounds, *T. repens* possesses antifungal, antibacterial, and insecticidal properties and enhances nitrogen fixation.

The flavonol contents of *T. repens* are different among different cultivars as studied by Hofmann et al. [83]. The summary of different cultivars is shown in Table 2, which showed that different cultivars of *T. repens* contain different flavonol contents in different parts of plants. For example, flavonol contents in leaves were 2–1700 mg/g, seeds 2.8–2000 mg/g, total above-ground materials were 20–2,210 mg/g, roots 208 mg/g, and flowers 66–481 mg/g. Min et al. [36] reported the prodelphinidin compound present in condensed tannins, a subclass of flavonols.

Table 3 shows the variation of flavonol contents at different experimental conditions. Besides, the table shows that the flavone content of *T. repens* was higher when grown in the field than in the glasshouse. Another class of flavonols, the condensed tannins, was also studied in *T. repens* representing high concentration in flowers as compared with the total above-ground material. Oleszek and Stochmal [85] reported that soyasapogenol B glycosides are the chemotaxonomic character of the Fabaceae family.

Isoflavonoids

Oleszek et al. [98] studied *Trifolium* species using thin layer chromatography (TLC) and HPLC techniques. They analyzed 57 *Trifolium* species for isoflavonoids and concluded that *T. repens* contains phenolic compounds (1–1.8% of dry mass) having a higher concentration of isoflavonoids among all phenolic compounds. Christiansen et al. [21] reported eight different isoflavones in *T. repens* such as sissotrin, biochanin A, genistein, genistein, formononetin, puerarin, daidzin, and daidzein. These compounds show different activities such as antifungal activity and antibacterial and reduce postpartum depression and skin aging [10, 40–45]. *T. repens* containing vestitone and 7,2',4'-trihydroxyisoflavone exhibit fungitoxic activities. Besides the aforementioned compounds, it also contains its subclass compounds such as coumestans and pterocarpanes, which act as antifungal, anti-*Helicobacter pylori*, anti-inflammatory, antiaging, and antihepatotoxic agents [46–50]. Generally, those plants which synthesize phytoestrogens or their derivatives are considered safe for the treatment of hormonal replacement therapy as compared with conventional therapy. Isoflavones and other plant derivatives such as coumestans, lignans, and stilbenes possess estrogenic properties [99]. Another study showed that isoflavones had mild estrogenic activity, but these compounds accrued in much more high folding

concentration even make 100-fold high concentration as compared with endogenous estrogen [100].

The literature reported that plant isoflavones play a significant role in the physiology of phytoestrogen in humans. Different researchers showed that isoflavones obtained from *Glycine max* (soy) are helpful in the physiological study of a phytoestrogen [100–102]. *T. repens* contain a lot of isoflavone compounds, and each type of compound exhibits a specific role in the biological system. Twenty-four phenolic compounds were identified in the aqueous extract of *T. repens*, among which the highest amounts present were of kaempferol-3-(caffeoyl diglucoside)-7-glucoside (983.7 µg/mL), followed by p-coumaroyl-4-glucoside (905.6 µg/mL) and daidzein-*O*-sulfate [103]. These compounds showed hepatoprotective properties.

The effects of isoflavones on a biological system of isoflavones such as biochanin A and genistein showed a strong phytoalexin behavior. Several isoflavones showed antifungal properties and estrogenic activity in ruminants. Table 2 presents antifungal, antibacterial, fungitoxic, anti-*Helicobacter pylori*, anti-inflammatory, antiaging, and antihepatotoxic properties of *T. repens* and their role in reducing postpartum depression and skin aging [103]. Coumestrol showed antibacterial activity to some strain, whereas coumestans generally exhibit phytotoxic activity. But coumestrol in *T. repens* showed contrasting results on the estrogenic properties and present more activity than isoflavones. In mouse or rat experiments, the estrogenic activity of coumestrol showed less activity as compared with the expected [10, 40–50]. There is a lack of literature on the study using isoflavones or their derivatives extracted from *T. repens* for the treatment of humans or animals. In addition, there is no experimental information available on the estrogenic activity of *T. repens*. In comparison to *Glycine max* which contain isoflavone compounds such as glycitein, daidzein, and genistein, *T. repens* contain at least seven isoflavone compounds, namely apigenin (4',5,7-trihydroxyflavone), isovitexin (apigenin-6-glucoside), saponarin (isovitexin-7-glucoside), luteolin (3',4',5,7-tetrahydroxyflavone), luteolin-4'-glucoside, luteolin-3',7-di-glucoside, and naringenin (4',5,7-trihydroxyflavanone) [92, 104]. Owing to the presence of widespread isoflavonoids, theoretically, it is ideal as a remedy for the treatment of different disorders.

Cyanogenic glucosides

Cyanogenic glycosides are the plant secondary metabolites, which act as natural plant toxins present in several plants including *T. repens*. In addition to plants, they are also found in bacteria and fungi. It has been reported that cyanogenic

Table 3: Contents of secondary metabolites found in healthy *Trifolium repens* under different conditions.

Compound	Content (mg/g dry wt)	Condition	Number of cultivars	References
Flavonols	559 ^a	No treatment, well watered	2 breeding lines, 4 + 3 ecotypes	[86]
	477 ^e	No treatment, droughted	2 breeding lines, 4 + 3 ecotypes	[86]
	1840 ^a	No treatment	1	[85]
	290–960 ⁱ	No treatment	2 breeding lines, 4 + 3 ecotypes	[83]
Kaempferol	Not found ^c	Arbuscular mycorrhizal	1	[82]
	n.d.–26.2 ^c	Arbuscular mycorrhizal	2	[10]
	297 ^e	No treatment, well watered	2 breeding lines, 4 + 3 ecotypes	[86]
	352 ^a	No treatment, droughted,	2 breeding lines, 4 + 3 ecotypes	[86]
	6 ^f	No treatment	1 experimental selection	[87]
	80–580 ⁱ	No treatment	2 breeding lines, 4 + 3 ecotypes	[83]
	2.8 ^a	No treatment	1	[88]
	139 ^e	Healthy glasshouse condition	1	[6]
Quercetin	<3 ^c	Arbuscular mycorrhizal	2	[6]
	208 ^c	Arbuscular mycorrhizal	1	[82]
	262 ^e	No treatment, well watered	2 breeding lines, 4 + 3 ecotypes	[86]
	125 ^e	No treatment, droughted	2 breeding lines, 4 + 3 ecotypes	[86]
	1160 ^a	No treatment	1	[85]
	450 ^f	No treatment	2 breeding lines, 4 + 3 ecotypes	[87]
	17 ^a	Healthy glasshouse condition	1	[10]
	14 ^e	Healthy glasshouse condition	1	[10]
6-Hydroxykaempferol	Not found ^c	Arbuscular mycorrhizal	1	[10]
Myricetin	179 ^c	Arbuscular mycorrhizal	1	[82]
Isorhamnetin	137 ^f	No treatment	1	[87]
Myricetin	5 ^a	No treatment	1	[10]
	2000 ^a	No treatment	1	[10]
5,6,7,8-Tetrahydroxy-4-methoxyflavonol	167 ^c	Arbuscular mycorrhizal	1	[82]
Flavones	326 ^c , l	With active rhizobial root nodules	1	[89]
4',7-Dihydroxyflavone	<2 ^e	Healthy glasshouse condition	1	[6]
	<2 ^e	Field healthy condition	1	[6]
3',4',7-Trihydroxyflavone	<2 ^e	Healthy glasshouse condition	1	[6]
	<2 ^e	Field healthy condition	1	[6]
Geraldone	<2 ^e	Healthy glass house condition	1	[6]
	<2 ^e	Field healthy condition	1	[6]
Acacetin	250 ^c	Arbuscular mycorrhizal	1	[82]
4',5,6,7,8-Pentahydroxyflavone	44.4 ^c	Arbuscular mycorrhizal	1	[82]
5,6,7,8-Tetrahydroxy-4'-methoxyflavone	Not found ^c	Arbuscular Mycorrhizal	1	[82]
	6,000–94,000 ^a	Fall/spring/summer	One-cycle experimental selection	[90]

Table 3: (continued)

Compound	Content (mg/g dry wt)	Condition	Number of cultivars	References
	12,000–30,000 ^f	Spring	One-cycle experimental selection	[10]
	30,000–79,000 ^f	Fall/spring/summer	One-cycle experimental selection	[90]
	0–12,100 ^l , II	Fall/spring/ summer	One-cycle experimental selection	[10]
	n.d.–600 ^e	Spring/early summer	1	[10]
	16,000–46,300 ^f	Spring/early summer	1	[10]
	1,600–11,600 ^l , II	Summer	1	[91]
	33,000 ^f	Summer	1	[10]
	60,000 ^l , III	Summer	1	[10]
	2,400 ^f		One-cycle experimental selection	[87]
	48 ^f		One-cycle experimental selection	[87]
Isoflavones	253 ^c	Field healthy condition	1	[92]
	354 ^d	Field healthy condition	1	[92]
	327 ^e	Field healthy condition	1	[92]
	213 ^f	Field healthy condition	1	[92]
	119 ^d		1	[10]
	27 ^e		1	[10]
	94 ^f		1	[10]
	100–600 ⁱ	Field healthy condition	4	[93]
Daidzein	500 ^g		1	[10]
	<2500 ^g		2	[10]
	0.3–40.6 ^c	Arbuscular mycorrhizal	2	[10]
	6 ^c	Summer	1	[78]
	4 ^d	Field healthy condition	1	[78]
	5 ^e	Field healthy condition	1	[78]
	1 ^f	Field healthy condition	1	[78]
	9 ^d		1	[6]
Pseudobaptigenin	5 ^e		1	[6]
	6 ^f		1	[6]
	<1–1 ⁱ	Field healthy condition	1	[6]
	42 ^c	Field healthy condition	1	[78]
Formononetin	669–1134 ^c	Arbuscular mycorrhizal	2	[6]
	4920 ^c , I	With rhizobial root	1	[89]
	900 ^g ,		1	[89]
	17–94 ^c , I	Healthy	2	[6]
	4–11 ^d , I	Healthy	2	[6]
	4–8 ^e , I	Healthy	2	[6]
	85 ^d		1	[94]
	7 ^e		1	[94]
	52 ^f		1	[94]
	90–570 ⁱ	Summer	4	[6]
	200 ^g		1	[6]
	220–920 ⁱ		32	[6]
	6 ^e		1	[6]
	0–800 ⁱ		3	[6]
Genistein	4.1–11.9 ^c	Arbuscular mycorrhizal	2	[6]
	nd ^c	Summer	1	[78]
	20 ^d		1	[94]
	5 ^e		1	[94]
	23 ^f		1	[6]
	5–60 ⁱ	Summer	4	[6]

Table 3: (continued)

Compound	Content (mg/g dry wt)	Condition	Number of cultivars	References
Biochanin A	300 ^a		1	[6]
	0–200 ⁱ		3	[6]
	2.4–5.5 ^c	Arbuscular mycorrhizal	2	[6]
	nd ^c	Summer	1	[78]
	5 ^d		1	[94]
Glycitein	2800 ^c	Summer	4	[6]
	0–600 ⁱ	Summer	1	[78]
	150 ^c	Summer	1	[78]
	282 ^d	Summer	1	[78]
	241 ^e	Summer	1	[78]
Calycosin	40 ^f	Summer	1	[78]
	55 ^c	Summer	1	[78]
	69 ^d	Summer	1	[78]
	82 ^e	Summer	1	[78]
	171 ^f	Summer	1	[78]
Pterocarpans	n.d. ^c	Summer	1	[78]
Medicarpin	17.0–76.6 ^c	Arbuscular mycorrhizal	2	[6]
	1–8 ^g , I	Healthy	2	[95]
	6–18 ^d , I	Healthy	2	[95]
	9–32 ^e , I	Healthy	2	[95]
Coumestans	<1–8.9 ⁱ		4	[6]
Coumestrol	<10 ⁱ		32	[6]
	<2 ^e	Healthy glass house condition	1	[6]
	<2 ^e	Field healthy condition	1	[6]
Trifoliol	<2 ^e	Healthy glass house condition	1	[6]
Cyanogenic glucosides (measured as potential of release of HCN)	53–2242 HCN ^e		7	[96]
	58–2205 HCN ⁱ		6	[94]
Linamarin	n.d.–2534 ⁱ	Spring	8	[97]
	1290 ⁱ		1	[97]
Lotaustralin	n.d.–4844 ⁱ	Spring	8	[97]
	3130 ⁱ		1	[6]
Saponins	3160 ^a		1	[6]
Cloversaponin I	1.5 ^w		1	[6]
Cloversaponin II	2.4 ^w		1	[6]
Cloversaponin III	1.72 ^w		1	[6]
Cloversaponin IV	20 ^w		1	[6]
Cloversaponin V	11.06 ^w		1	[6]
Soyasaponin I	1120 ^a		1	[6]
Azukisaponin II	12.53 ^w		1	[85]
Astragaloside VIII	860 ^a		1	[85]

Different letters in the table represent the following: a, in seeds; b, in shoots; c, in roots; d, in the stem; e, in leaves; f, in flowers; g, in leaves and stem; i, above-ground materials; w, in the whole plant; I, the content determined in fresh weight, but the final calculation was in dry weight, assuming a water content of 80%; II: assuming that there are negligible condensed tannins in other plant parts; III: in flower petals; n.d.: not detected.

glucosides such as linamarin and lotaustralin were present in *T. repens* [51, 52, 105, 106]. The literature reported significant variation in the concentration of cyanoglucoside content in different cultivars as shown in Table 2. It was observed that leaves contained a high amount of cyanogenic

glycosides which was 16–2242 µg/g. Linamarin and lotaustralin cyanogenic glycosides always occur together in *T. repens*, where the concentration of lotaustralin was always higher than linamarin. Besides, cyanogen synthesis depends on different factors such as season, light intensity, water

availability, and temperature. There was no effect of fungal infection on HCN production in *T. repens* [10, 107]. Foo et al. [87] reported that *T. repens* contained proanthocyanidins, phenolic compounds of lower molecular weight, prodelphinidin, epigallocatechin, gallic acid, gallic acid-(4 α -8)-epigallocatechin, and *cis*- and *trans*-p-coumaric acid-4-O- β -glucopyranoside. Two cyanogenic glucosides, that is, linamarin and lotaustralin, have also been identified in *T. repens* in Japan [108].

Cyanogenic production in *T. repens* is highly effective against fungal infection, herbivores, and weevils. Literature supports that HCN poisoning is highly susceptible to ruminants as compared to monogastric organisms. The variation in action is due to the variation in the degradation and absorption. Generally, those varieties of *T. repens* are considered safe as they synthesize low amount of HCN [10]. Although HCN content in high doses is toxic to *T. repens*, its amount is not significant to cause toxicity. Studies [51, 52] reported the antifungal and antidiabetic activity of cyanogenic glycosides.

Saponins

Saponins are the plant secondary metabolites which are the vast group of glucosides synthesized by several groups of plants. The plants containing saponins are used for medicinal purposes and often used for a specific therapeutic function [109]. *T. repens* also showed the presence of saponins. The compounds present in saponins are soyasaponin I methyl ester, soyasaponin I, soyasaponin II methyl ester, azukisaponin II methyl ester, cloversaponin I–V methyl ester, astragaloside VIII. Owing to the presence of these compounds, *T. repens* exhibit different biological activities such as antifungal activity, hepatoprotective effect, anti-inflammatory and antioxidant properties [53–56].

About nine saponins have been reported including four known and five new compounds by Sakamoto et al. [110] using the HPLC method for identification and quantification. The reported saponins in *T. repens* are cloversaponin, methyl ester, cloversaponin methyl ester, cloversaponin methyl ester, cloversaponin methyl ester, and cloversaponin methyl ester.

Saponin showed antifungal activity, hepatoprotective effect, anti-inflammatory, and anti-oxidant health-promoting activities and is toxic to nematodes [53–56, 111, 112]. Class soyasaponin I shows a toxic effect on the plant-parasitic nematode. Adel et al. [113] described that six saponins in *T. repens* including aglycones, soyasapogenol B, and soyasapogenol E are less active than soyasaponin I against the insect.

Lipids and fatty acids

In *T. repens*, the free dominant free fatty acids are linolenic acid and triacontanol as the principal free fatty acid reported by Maffei [114]. Besides, the hydrocarbon C29 and C31 were noted in large quantity. Maffei [114] reported the concentration of fatty acid that *T. repens* contain using gas chromatography (GC) and concluded that *T. repens* contain different fatty acid concentrations such as C12:0 (0.7 mg/g of DM), C14:0 (0.13 mg/g of DM), C16:0 (5.22 mg/g of DM), C16:1 (0.88 mg/g of DM), C18:0 (0.84 mg/g of DM), C18:1 (1.46 mg/g of DM), C18:2 (4.62 mg/g of DM), C18:3 (6.52 mg/g of DM), and total free fatty acids (29.76 mg/g of DM).

Another study was conducted on *T. repens* cultivation under different stress conditions, and the concentration of fatty acid at each cultivar was calculated. There was an average amount (3.34 mg g⁻¹ of dry mass [DM]) of palmitic acid at different environmental stress conditions. Similarly, the average amount of C16:1 is 0.62 mg/g of DM, C18:0 (0.31 mg/g of DM), C18:1 (0.73 mg/g of DM), C18:2 (2.34 mg/g of DM), C18:3 (4.7 mg/g of DM), which was measured using GC techniques [115]. No data are available to check the biological effect of plant fatty acids from *Trifolium* on humans and animal models.

Volatile compounds

In *T. repens*, comparatively little evidence is available about the volatile compounds. Kami [116] conducted a study for the analysis of volatile compounds produced by *T. repens* using the steam distillation method. The author isolated 80 compounds containing phenols, acids, ketones, aldehydes, alcohols, hydrocarbons, and esters. Another study was conducted by Tan et al. [117] on the extraction of honey components from *T. repens*. In this study, the components of the extract were methylated for identification using GC and mass spectrometry. About 61 volatile compounds including the compounds present in higher quantity such as dimethyl succinate, methyl 2-methoxybenzoate, methyl caprylate, methyl benzoate, methyl linoleate, pentacosane, dimethyl-2-decenedioate, methyl-3,5-dimethoxybenzoate, methyl-3,4-dimethoxybenzoate were identified (Figure 2).

Antioxidant activity of *Trifolium repens*

As discussed previously, *T. repens* are rich in important natural antioxidants; thus, they possess antioxidant activities. These antioxidants protect the plant by itself

Figure 2: Structure of the important volatile compounds present in *T. repens*.

against the different types of external stresses [118–120]. The flower extract showed strong radical scavenging activity in both DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) tests with IC₅₀ values of 10.3–180.8 and 21.4 µg/mL, respectively [121–123]. In addition to bioactive compounds, *T. repens* explants also showed four different forms of natural antioxidant enzymes such as superoxide dismutase [124]. Shang et al. [125] reported the antioxidant activity of four different extractions, that is, hot water extraction (HWE), ultrasonic-assisted extraction (UAE), enzyme-assisted extraction (EAE), and ultrasonic-enzyme-assisted extraction (UEAE). These authors showed that EAE showed stronger DPPH scavenging ability compared with HWE, UAE, and UEAE. The higher content of uronic acid in EAE might contribute to its higher DPPH radical scavenging activity. The highest DPPH (61.99%) and ABTS (99.0%) radical scavenging activity of EAE was detected at 1 mg/mL, respectively. A recent study [75] showed the antioxidant activities (DPPH and ABTS assays) of the different extracts (hexane, chloroform, ethanol, methanol, and water varied significantly) as shown in Table 4. In the DPPH antiradicals study, chloroform fractions exhibited the highest scavenging effects (87.4%) at the highest tested concentration of 1 mg/mL and IC₅₀ of 42 µg/mL. Whereas, ethanolic fraction showed 72.5% inhibition of DPPH radicals at 1 mg/mL concentration and IC₅₀ of 110 µg/mL. These two were the most potent fractions among all samples. Other fractions exhibited moderate and concentration-dependent inhibition. Similarly, chloroform and ethanolic fractions exhibited strong antioxidant potentials against ABTS radicals with percent inhibitions of 89.7 and 75.7 at 1 mg/mL, respectively. The IC₅₀ values for chloroform and ethanolic fractions were 25 and 98 µg/mL, respectively.

Role of white clover–derived compounds in cancer

As discussed previously, different phytochemicals of *T. repens* have different pharmacological properties (Figure 3), and the plant is also known to have anticancer properties. Abnormal or high production of reactive oxygen and nitrogen species results in the formation of cancer development, inflammation, a neurodegenerative disorder, cardiovascular defect formation, and autoimmune diseases [126, 127]. Several studies revealed that isoflavones play a key role in the antioxidant system [128]. The antioxidant activity of the white clover–derived compound isoflavone has not been recorded [129].

For this purpose, a pilot study was conducted as described by Campbell et al. [130]; however, this study was on the red clover from which 86 mg/day of isoflavones was derived and administered to mice, which showed a very little effect on the marker of antioxidant. But in another study by Mu et al., long-term administration of formononetin (*T. pratense*-derived) was performed [131]. Mice were selected for the study and divided into five groups, control group; low-dose formononetin group, which was administered with 0.05 g/kg per day; high-dose formononetin group, 0.5 g/kg per day; sham-operated group; and ovariectomized group. In the end, a significant increase was noted in the activities of enzymes such as glutathione peroxidase, superoxide dismutase, and catalase and reduction in lipid peroxidation. The antioxidant properties of *T. repens* may be due to the presence of flavonoids, isoflavonoids, clovamide, saponins, and other valuable compounds present in it.

Phytoestrogens present in the plant may affect angiogenesis and do function as chemopreventive agents.

Table 4: Antioxidant activities of different fractions of *T. repens* [75].

Sample fraction	Concentration (µg/mL)	% ABTS scavenging (mean ± SEM)	ABTS IC ₅₀ (µg/mL)	% DPPH scavenging (mean ± SEM)	DPPH IC ₅₀ (µg/mL)
Hexane	1,000	61.2 ± 0.5***	470	66.0 ± 1.1***	290
	500	55.3 ± 0.5***		60.0 ± 0.0***	
	250	51.4 ± 0.6***		54.7 ± 1.6***	
	125	45.3 ± 0.5***		47.8 ± 0.3***	
	62.5	41.4 ± 0.5***		44.9 ± 0.4***	
Chloroform	1,000	87.4 ± 0.5 ^{ns}	42	89.6 ± 1.3 ^{ns}	25
	500	82.3 ± 0.4 ^{ns}		84.4 ± 0.4 ^{ns}	
	250	75.6 ± 0.4 ^{ns}		78.2 ± 1.4 ^{ns}	
	125	67.5 ± 0.7 ^{ns}		72.2 ± 1.2 ^{ns}	
	62.5	61.3 ± 0.6 ^{ns}		65.0 ± 0.4 ^{ns}	
Ethanol	1,000	72.5 ± 0.6***	110	75.7 ± 0.9*	98
	500	66.4 ± 0.5***		71.0 ± 0.3*	
	250	57.4 ± 0.6***		65.2 ± 1.2**	
	125	52.3 ± 0.5***		59.9 ± 1.3**	
	62.5	45.3 ± 0.6***		51.9 ± 0.8**	
Methanol	1000	67.3 ± 0.6***	230	69.7 ± 0.6***	225
	500	61.3 ± 0.6***		65.5 ± 0.7***	
	250	56.3 ± 0.5***		57.3 ± 0.6***	
	125	48.5 ± 0.4***		51.4 ± 0.5***	
	62.5	42.3 ± 0.5***		44.4 ± 0.6***	
Water	1000	51.5 ± 0.7***	1900	54.9 ± 2.0***	1350
	500	45.2 ± 1.0***		47.1 ± 1.0***	
	250	37.5 ± 0.6***		42.6 ± 1.6***	
	125	32.9 ± 1.0***		35.7 ± 0.6***	
	62.5	27.6 ± 0.5***		29.0 ± 0.8***	

All experiments were performed in triplicate, and results were expressed as mean ± SEM. Values are significantly different in comparison to those of ascorbic acid, that is, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ analyzed at the same tested concentration; ns: values are not significantly different as compared with those of the positive control.

Downregulation of genes and mRNA of that protein occurs, which is involved in angiogenesis due to isoflavones such as daidzein and genistein. In addition, the formation of antiangiogenic factors may start the isoflavone-mediated upregulation. Krenn and Paper [132] demonstrated that nonmethylated isoflavones such as genistein and daidzein possess high antiangiogenic activity than the methylated compounds such as biochanin A and formononetin. Although such types of literature are not available on *T. repens* species, the compound which is present in *T. repens* may act as that in red clover.

Another study was conducted for the prevention of prostate cancer using the red clover. In *in vitro* state, isoflavones derived from the red clover suppress the proinflammatory effects in human prostate cancer-derived stromal cells [133]. Jarred et al. [134] also carried out a study to find out the effect of isoflavonoids derived from the red clover in prostate cancer prevention. They concluded that isoflavones at the dose of 160 mg per day may lead to halting the development of prostate cancer by inducing apoptosis in low

to moderate grade cancer tumors [134]. The conclusion from different research showed that isoflavonoids such as formononetin possess anticancer properties. There is a lack of literature on the anticancer activities in *T. repens*. But the compound present in the red clover is also present in *T. repens*; therefore, the *T. repens* may also exhibit different anticancer properties. The cytotoxicity of *T. repens* was studied in A549 cell lines, which showed the efficacy of *T. repens* [135], suggesting further studies. A recent study was reported by Sarno et al. [136] of the antitumor activity of *T. repens* on a panel of liquid and solid cancer cell lines, including colon cancer HCT-116, breast cancer MCF7, lung cancer A549, and hepatocellular carcinoma HepG2 cells and an effect was observed only in chronic myeloid leukemia (CML) cells. They reported that *T. repens* blocks the proliferation of cancer cells. The block of cell growth was associated with a total inhibition of BCR-ABL/STAT5 and activation of the p38 signaling pathways. They also found that these strong cytotoxic effects did not occur in normal cells. They suggested that the development of novel compounds derived from

Figure 3: Schematic representation of traditional and pharmacological application of *T. repens*.

phytochemical molecules present in *T. repens* might lead to the identification of new therapeutic agents active against chronic myelogenous leukemia.

Anti-inflammatory activity

The *Trifolium* species such as the red clover have been reported to possess anti-inflammatory activity in carrageenan-induced rat paw edema test [137]. However, there is a lack of literature regarding the anti-inflammatory activity of *T. repens*. The presence of important flavonoids may contribute to the activity. However, in comparison to

other species, among the Spanish plants, *T. repens* showed very weak inhibitory activity of cyclooxygenase and platelet aggregation factor-induced exocytosis [138]. Recently, Chen et al. [32] reported the anti-inflammatory activities of dichloromethane extracts, and three isolated constituents daphnoretin, 2H-1-benzopyran-2-one-7-hydroxide, and hydroxy-3-(4-methoxy-phenyl)-chromen-4-one were evaluated with lipopolysaccharide (LPS)-induced RAW 264.7 macrophages at a series of concentrations. RAW 264.7 cells were pretreated with LPS (0.5 mg/mL) in the presence or absence of the constituents for 24 h, followed by the collection of the resulting cells for Western blot analysis. These authors showed that expression of iNOS and COX-2 in LPS-induced

RAW cells was suppressed by the pretreatment with three isolated constituents. In conclusion, *T. repens* can be effectively used to treat inflammation.

Conclusion and future perspectives

The review summarizes all the essential information about *T. repens*, their phytochemistry, and their biological effects. *T. repens* is one of the most important vegetables, a fodder, food, and a medicinal plant. The plant is rich in important flavonoids, isoflavones, phenolic acids, glycosides, monosaccharides, proteins, essential fatty acids, tocopherols, and carotenoids. *T. repens* is used for anti-inflammatory, antiseptic, analgesic, antioxidant, antirheumatic ache, and antimicrobial purposes. However, substantial information is still missing about the pigment composition, phenolic compounds, carotenoids, and biological functions of *T. repens*, especially in the animal models.

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