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Committee on Herbal Medicinal Products (HMPC)

Assessment report on *Juglans regia* L., folium

Based on Article 16d(1), Article 16f and Article 16h of Directive 2001/83/EC as amended (traditional use)

Draft

Herbal substance(s) (binomial scientific name of the plant, including plant part)	*Juglans regia* L., folium
Herbal preparation(s)	*Juglans regia* L., leaves cut and dried b) Herbal preparations Comminuted herbal substance
Pharmaceutical forms	Comminuted herbal substance for preparation of decoction for cutaneous use
Rapporteur	
Assessor(s)	

Note: This Assessment Report is published to support the release for public consultation of the draft Community herbal monograph on *Juglans regia* L., folium. It should be noted that this document is a working document, not yet edited, and which shall be further developed after the release for consultation of the monograph. Interested parties are welcome to submit comments to the HMPC secretariat, which the Rapporteur and the MLWP will take into consideration but no 'overview of comments received during the public consultation' will be prepared in relation to the comments that will be received on this assessment report. The publication of this draft assessment report has been agreed to facilitate the understanding by Interested Parties of the assessment that has been carried out so far and led to the preparation of the draft monograph.

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1. Introduction

The herbal substance of European walnut (*Juglans regia* L.) leaves is mentioned in several well-known handbooks and sources such as Madaus (1938), Deutschen Arzneibuch (1926), Wren (1975), Ożarowski (1976), Miller and Murray (1998), Blumenthal (2000), Wyk and Wink 2004, Martindale (2009), Wichtl (1994; 2004), PDR for Herbal Medicines (Gruenwald et al.(ed) 2000; 2004), German Comission E Monograph, Gehrman et al. (2005) and Natural Medicines Comprehensive Database (2009).

The herbal substance is described as the dried entire –margined leaflets freed from the rachis with faintly aromatic odour and astringent, somwhat bitter and harsh taste (Wichtl , 2004). By long preservation the leaves become brown and lose their aroma. *Juglans regia* leaves contain ca. 10% tannins (elegitannins). Juglone is unstable and in dry leaves is present only in traces.

European walnut belongs to the family of Juglandacae which contains about 50 species in 8 genera. The deciduous tree native in Southeastern Europe, is now cultivated and distributed in North temperate and subtropical regions of Europe, Africa, Asia and America. It is native in a region stretching from the Balkans eastward to the Himalayas and southwest China. The largest forests are in Kyrgyzstan, where trees occur in extensive, nearly pure walnut forests at 1,000–2,000 m altitude. Possibly native in Greece and elsewhere in the Balkan penninsula; widely cultivated and locally naturalized in Europe (Flora Europea 2011). Its Latin name *Juglans* originates from the Latin *Jupiter* and *glans* (acorn) meaning “Jupiter nuts”.

Tannins

Leafs contains approximately 10% tannins of the ellagitannins type (Blumenthal 2000). According to DAC not less than 2.0%, calculated as pyrogallol (Wichtl, 2004).

Naphtalene derivatives

Naphthoquinones, oxygen-derivatives of naphthalene. The most known constituent is juglone (5-hydroxy-1,4-naphtoquinone) which occurs in fresh plant (leaf, stain) as glycoside of reduced form: 4β-D-glucoside of α-hydrojuglone=4β-D-glucoside of 1,4,5-trihydroxynaphthalene (2% in the stain, 0.6% in the leaves), but also in free state, particularly in the epicuticular leaf wax (to about 30%), (Wichtl 2004, Evans 2000,Hazra et al. 2004; Prasad and Gülz 1990).

This glucosidic form is decomposing by hydrojuglone-β-D-glucopyranoside-β-glucosidase to juglone (Morant 2008). Juglone is unstable and easily polymerized into yellow, brown and black pigments, so in the older leaves or dry leaves is present only in trace amounts. As dried leaves were used for extract preparation, juglone was not detected, which means that this compound is not suitable for use in quality control of dry plant (Amaral et al. 2004; Babula et al. 2009; Gîrzu et al. 1998; Wichtl 2004; Wójcik 1984).

Flavonoids

Leaf contains about 3.4% of flavonoids (Wichtl 2004, Carnat et al. 1993), especially quercetin, hyperoside=quercetin 3-*O*-galactoside (0.6% according to Carnat et al. 1993), and 0.2-0.6% of quercitrin=quercetin 3-*O*-rhamnoside (Wichtl 2004), kaempferol and kaempferol 3-*O*-arabinofuranoside have been also isolated (Liu et al. 2004).

Several flavonoids (quercetin 3-galactoside, quercetin 3-arabinoside, quercetin 3-xyloside, quercetin 3-rhamnoside and two other partially identified 3-pentosides of quercetin and kaempferol in walnut

cultivars, collected at different times from walnut grown in Portugal, were also detected (Amaral et al. 2004). Quantification of flavonoid compounds in the walnut grown in Portugal cultivars performed by HPLC-DAD, revealed that flavonols were always the major compounds, varying between 54.8% and 62.9% of total phenolics, whereas quercetin 3-galactoside was always the major constituent (Pereira et al. 2007). They identified another two hydroxycinnamic acid dervatives, the 5-0-caffeoylquinic and p-coumaric acids (Pereira et al. 2007).

According to Fr Ph, 10th Ed. Juglandis folium contains not less than 2.0% of total flavonoids (Bruneton 1999).

Phenolic acids

Previous data revealed the presence of phenolic acids: *p*-hydroxybenzoic, vanillic, genistic, protocatechuic, *p*-coumaric, caffeic, ferulic, gallic, chlorogenic (3-caffeoylquinic) acids (Łuczak et al. 1989) and neochlorogenic (5-caffeoylquinic acid) (Blumental 2000). Data concerning quality of walnut leaves samples from six different cultivars grown in Portugal not confirmed the presence of above compounds with the exception of chlorogenic acid (Amaral et al. 2004), which was the major phenolic acid (7.17-22.3%), together with 3-*O*-coumaroylquinic and 4-*O*-coumaroylquinic acids (Amaral et al. 2004). Moreover recent publication of the same group described significant differences in the phenolics composition from nine different cultivars along three consecutive years (3-caffeoylquinic, 3-*p*-coumaroylquinic and 4-*p*-coumaroylquinic acids, quercetin 3-galactoside, quercetin 3-arabinoside, quercetin 3-xyloside, quercetin 3-rhamnoside, a quercetin 3-pentoside derivative and a kaempferol 3-pentoside derivative) (Amaral et al. 2008). According to authors suggestion the differences observed can be related to climatic conditions.

Additionally the presence of neochlorogenic and *p*-coumaric acids in the analysed cultivars were detected; all samples exhibited the same phenolics profile, whereas 3-caffeoylquinic acid was the major constituent (about 19.7%) and *p*-coumaric acid was the minor compound, representing ca. 1.4% of total phenolics (Pereira et al. 2007).

The total phenolic content in one of the above cultivars measured in methanolic and petroleum ether extracts were 94.39±5.63 and 92±1.40 mg GAE/g, respectively (Carvalho et al. 2010).

Volatile oil

The leaf contains trace amounts of essential oil (obtained by the Tenax trapping method) of the order of 4 ppm, with monoterpene and sesquiterpene hydrocarbons. Monoterpenes are represented by: (E)-β-ocimene (12%), β-pinene (11%), limonene (10%), with traces of sabinene, α-pinene, myrcene, and linalool, whereas sesquiterpenes by caryophyllene (15%), germacrene D (13%), with minor amounts of (E)-β-farnesene and α-farnesene (Buttery et al. 1986).

Nahrstedt et al.(1981) received 26 terpenoid substances from steam distilled leaves of European walnut. The substances identified were: 21 monoterpenes, two sesquiterpenes, one diterpene, two compounds of terpenoid origin and eugenol. The steam distilled yield comprehends more sesquiterpenes and substances derived of esters of fatty acids, with the main substance – β-eudesmol.

Liu et al. (2004) performed analysis of an essential oil extracted from *Juglans regia* leaves by hydrodistillation. By use of GC-MS method they identified 20 components: terpenoids (84.89%), aromatics (3.9%) and esters (1.34%).

Farag (2008) described detailed results of volatile compounds in the leaves from selected plants of Juglandaceae family, *Juglans regia* included where with use of GC-MS method Were identified

sesquiterpenes (47%), with germacrene D in highest amount (28%). High levels of monoterpenes were also found in walnut leaves (24%) as well as high amount of methyl salicylate (17%).

Other compounds

Ascorbic acid (0.85-1.0%) (Wichtl 2004).

The leaf also contains: cyclitols (11.2%), mucilage (7.6%), calcium (1.9%), potassium (1.4%) and 8.7% of ash (in brackets are avarage levels of 15 samples of dry leaves) (Carnat et al 1993).

The surface waxes from the leaves of *Juglans regia* exhibited presence of primary alcohols (41.6 %), hydrocarbons (3.0 %), esters (3.5 %), aldehydes (5.5 %) and fatty acids (8.4 %) (Prasad & Gülz 1990).

From the fresh leaves progesterone was obtained with yield of 0.00010% w/w (Pauli et al.2010).

1.1. Description of the herbal substance(s), herbal preparation(s) or combinations thereof

- Herbal substance(s)

Leaves cut and dried

- Herbal preparation(s)

Comminuted herbal substance(10% tannins)

- Combinations of herbal substance(s) and/or herbal preparation(s) including a description of vitamin(s) and/or mineral(s) as ingredients of traditional combination herbal medicinal products assessed, where applicable.

The herbal substance is also available in combination products. Main plants used in combination are: Agropyron repens, Foeniculum vulgare, Foeniculum vulgare subsp. dulce, Hyssoppus officinalis, Juniperus communis, Mentha piperita, Phaseolus vulgaris, Plantago lanceolata, Potentilla anserina, Quercus robur, Rhamnus frangula, Sambucus nigra, Taraxacum officinale, Thymus vulgaris.

1.2. Information about products on the market in the Member States

Regulatory status overview

Member State	Regulatory Status				Comments
Austria	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	
Belgium	☐ MA	☐ TRAD	☐ Other TRAD	☒ Other Specify:	The herbal substance is only available in combination products
Bulgaria	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	
Cyprus	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	
Czech Republic	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	
Denmark	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	
Estonia	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	
Finland	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	
France	☐ MA	☐ TRAD	☐ Other TRAD	☒ Other Specify:	The herbal substance is only available in homeopathic products
Germany	☒ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	
Greece	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	
Hungary	☐ MA	☐ TRAD	☒ Other TRAD	☐ Other Specify:	The herbal substance is only available in combination products
Iceland	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	
Ireland	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	
Italy	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	
Latvia	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	
Liechtenstein	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	
Lithuania	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	
Luxemburg	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	
Malta	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	
The Netherlands	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	
Norway	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	
Poland	☐ MA	☒ TRAD	☐ Other TRAD	☐ Other Specify:	
Portugal	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	
Romania	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	
Slovak Republic	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	No products
Slovenia	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	
Spain	☐ MA	☒ TRAD	☐ Other TRAD	☐ Other Specify:	
Sweden	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	No products
United Kingdom	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	

MA: Marketing Authorisation

TRAD: Traditional Use Registration

Other TRAD: Other national Traditional systems of registration

Other: If known, it should be specified or otherwise add 'Not Known'

This regulatory overview is not legally binding and does not necessarily reflect the legal status of the products in the MSs concerned.

Table 1. Products on the market

Active substance	**Indication**	**Posology**	**Period of medicinal use**
Herbal tea			German Standard (WEU, Marketing Authorization*, Germany)
Comminuted herbal substance Herbal tea as decoction	Traditionallly used: Mild, superficial inflammation of the mucosa and skin, excessive perspiration of hands and feet	Adults: Topical use	At least since 1963, Poland
Comminuted herbal substance Herbal tea as decoction	Traditionallly used: Inflammation of the mucosa and skin; small wounds	Adults: Oromucosal use for gargles as a mouthwash Topical use	At least since 1973 (TU, authorised**, Spain)

**For the sake of completeness, all preparations for which marketing authorisations for traditional use have been granted (with reference to former national regulations) are mentioned, regardless of the fact that some of them are not in accordance with current community law (as defined in directive 2004/24/EC). Traditional preparations were authorised in 10-50% of well-established use doses when in parallel the same preparations were authorised under well-established use.*

Additional information for the WEU herbal tea from *Juglans regia* folium authorized in Germany.
**Additional comments: The herbal substance is not available in combination products.
Risks (adverse drug effects, literature)

Information on other products

Hungary

The herbal substance is only available in combination products: >5

Main plants used in combination are: *Agropyron repens*, *Foeniculum vulgare*, *Foeniculum vulgare* subsp. *dulce*, *Hyssoppus officinalis*, *Juniperus communis*, *Mentha piperita*, *Phaseolus vulgaris*, *Plantago lanceolata*, *Potentilla anserina*, *Quercus robur*, *Rhamnus frangula*, *Sambucus nigra*, *Taraxacum officinale*, *Thymus vulgaris*.

Posology For topical use: apply on affected region at least in morning and in evening, but not more than 5 times daily.
For oral use: one level tablespoon (approx. 3.5 g) of tea mixture to one cup (0.2 litres) of hot water. Drink 1 – 3 cups of lukewarm tea daily.
Indications
Herbal tea orally in predisposition for diabetes and against obesity,
Topically against dermatomycosis.
Preparations (kind of extract, extraction solvent, DER)

1) Tincture (1:10.8 – 11.8) 19.03 g/20 ml (1.5 g Plantaginis lanceolatae folium, 1.5 g Anserinae herba, 1.0 g Hyssopi herba, 1.0 g Thymi herba, 1.0 g Quercus cortex pulv., **0.68 g Juglandis folium**/ 100 ml of tincture) (Since 1997)

2) Herbal tea mixture: (1.05 g Phaseoli legumen, 0.52 g Taraxaci radix, 0.52 g Maydis stigma, 0.35 g Juniperi p[seudo-fructus, 0.35 g Mentahae pip. Folium, **0.35 g Juglandis folium**, 0.35 g Graminis rhizoma/ 1 tablespoon [3.5 g]) (Since 1988)

3) Herbal tea mixture: (0.6 g Sambuci flos, 0.84 g Foeniculi dulcis fructus, 0.65 Graminis rhizoma, **0.42 g Juglandis folium**, 0.42 g Menthae piperitae folium, 0.21 g Juniperi pseudo-fructus, 0.105 g Frangulae cortex/ 1 tablespoon [3.5 g]) (Since 1988)

4) Herbal tea mixture in filters: 0.25 g Sambuci flos, 0.23 g Foeniculi fructus, 0.19 g Graminis rhizoma, **0.12 g Juglandis folium**/ filter [1 g]) (Since 1999).

Hungarian name for *Juglans regia*: diófalevél

Belgium

The herbal substance is present only in multicomponent teas (containing >6 herbal substances each, teas were authorized in 1961 - 63 and should be reoriented under TU scheme)

1.3. Search and assessment methodology

Databases assessed up to March 2011:

Science Direct, PubMed, Embase, Medline, Academic Search Complete, Toxnet

Search terms: *Juglans regia* leaves, European walnut, juglone

2. Historical data on medicinal use

2.1. Information on period of medicinal use in the Community

Paleobotanical data testify for vegetation history of *Juglans regia* in southern Europe, Syria, China, Himalaya and in Central Asia (Kyrgyzstan) of the last 6800 years, where walnut possibly outlived the last glaciation (Beer et al. 2008). Analysis of climate shows that spread of walnut is favourably correlated with climate warming Loacker et al. 2007).

Interesting discovery of walnut shell used as necklace is described by Klichowska (1971) from archeological excavations of cemetery of Roman influence period in Pruszcz Gdański.

Recorded paleobotanical data with reconstruction of vegetational history confirmed cultivation of *Juglans regia* throughout the past 6300 years in northeastern region of Italy (Lago della Costa) (Kaltenrieder et al. 2010).

In Mediterranean countries *Juglans regia* leaves use for medical purposes is a common tradition.

According to ethnobotanical data decoction of *Juglans regia* leaves is contemporary commonly used in Iberian Peninsula in traditional folk medicine as in mouthwash as dental antiseptic and against pharyngitis. Oral administration is used against renal stones and obesity (Agelet and Valles 2001; Blanco et al. 1999; Bonet et al. 1999),

Both in Bulgaria and Italy walnut leaves are traditionally used as astringent and anti-inflammatory remedy (Leporatti and Ivancheva 2003).

The leaves of *Juglans regia* containing repellent and antiparasitic substances were set in wheat or in lentils, and in sacks containing other cereals or legumes. The leaves were placed in hen-coops to remove lice, a decoction of leaves was administered to horses as vermifuge and were used to aromatise and to preserve cheeses in central Italy. In southern Italy fresh leaves were also applied topically to reduce the swelling associated with varicose veins in (Quave et al. 2008). For treatment of burns and to remove warts and pimples, poultices and ointments were applied (Guarrera 1999; 2003;2005a; Guarrera et al. 2005b).

In Sardinia *Juglans regia* leaves infusions are used in helminthiasis (Bruni et al. 1997).

In Turkey walnut leaves are frequently externally used against fever in sun stroke in acne, and as antifungal agent or to alleviate rheumatic pain (Erdemoglu et al. 2003; Kültür 2007).

Traditional use of *Juglans regia* as anthelmintic remedy is reported not only in Europe, but also on other parts of the world, as Indo-Pakistan subcontinent (Akhtar et al. 2000).

In Lebanon *Juglans regia* leaves are traditionally used as remedies in rheumatic and neuralgic complaints mainly as compresses and cataplasms (El Beyrouthy et al. 2008)

In northern and in south-eastern Morocco and in Egypt *Juglans regia* leaves and bark are used in oral hygiene, in skin infections and rashes and for hypertension treatment (El-Hilaly et al. 2003; Gazi 1986; Mouhajir et al. 2001; Tahraoui et al. 2007)The walnut tree has a long history thousands of years of traditional medicinal use, to treat a wide range of complaints (Bruneton, 1993).

Its use is mentioned in the ancient times. Walnut preparations were used during the Middle Ages mainly as a remedy for various skin ailments and were mentioned by Lonicerus (1564) and Matthiolus (1501-1577) Its therapeutic activity is presented by Schimpfky (1900) and in Madaus “Lehrbuch der Biologischen Heilmittel” (1938). The traditional use of *Juglans regia* in different diseases has been thoroughly documented in several handbooks and in folk tradition.

The leaves are contemporarily targeted to skin ailments as an anti-inflammatory, astringent and depurative. However in the past they were used as anthelmintic, treatment of dyspepsia, diarrhea, chronic coughs, asthma. The leaves are also used to treat skin disorders: acne (Magin et al. 2006; Reuter et al. 2010), eczema, herpes, scrofoulus disease, in excessive perspiration of the hands and feet and slow healing wounds as a wash, compress or poultices). Its astringent activity is assigned to the tannin content (Ożarowski 1976; Ożarowski and Jaroniewski 1987; Blumenthal 2000, Wren 1975, Weiss and Fintelman 2000; Wichtl 2004, Wyk and Wink 2004, Frohne 2006).

2.2. Information on traditional/current indications and specified substances/preparations

Information on indications from products on the market

Juglandis folium (herbal substance):
Traditionally used in mild, superficial inflammation of the mucosa and skin and excessive perspiration of hands and feet as astringent and depurative.

Information on traditional indications from literature

Commission E Monograph. *Juglans regia* L.,(Bundesanzeiger No 101, published June 1, 1990).
Juglandis folium leaves for: Mild, superficial inflammations of the skin; excessive perspiration, e.g. of the hands and feet.

Herbal Drugs and Phytopharmaceuticals (Wichtl, 2004)
Juglandis folium leaves for:
Topical route: In acne, eczema, scrophula, pyodermia, ulcers, excessive perspiration in the feet and hands.
Oral route: In folk tradition in gastrointestinal catarrhs, anthelmintic and as a “blood purifying remedy”.

Herbal Medicinals, Miller & Murray (1998).
Juglandis folium leaves for: Atopic dermatitis (exudative phase)

Herbal Medicine, (Weiss & Fintelman, 2000).
Juglandis folium leaves for: Dermatitis

Lehrbuch der Biologischen Heilmittel (Madaus, 1938)
Juglandis folium leaves for: Scrofulosis, hyperhidrosis, acne, herpes, crusta lactea, exanthema, eczema, tinea capitis.

Medicinal Herbs: A Compendium (Gehrman et al. 2005)
Juglandis folium leaves for: In mild, superficial skin inflammations; excessive perspiration, for example, of hands and feet.

Medicinal Plants of the World, Wyk & Wink, 2004)
Juglandis folium leaves for: Skin ailments: acne, eczema, fungal infections, inflammations, sunburn, perspiration, itchy scalp and ulcers. Decoctions are traditionally used against bedbugs and lice.

PDR for Herbal Medicines, Gruenwald et al.(ed.) (2000)
Juglandis folium leaves for: Mild, superficial inflammation of the skin, excessive perspiration.

Ziołolecznictwo, Ożarowski, (1976)
Juglandis folium leaves for: Mouthwash in stomatitis and inflammatory conditions of the throat, acne, neurodermatitis, eczema, hyperhidrosis.

Assessors' comment

On the basis of the information on traditional and current indications, data on clinical efficacy (see section 4.2) and the requirements for specified conditions of use to ensure a safe use, the following therapeutic indications are recommended for *Juglans regia* leaves included in the monograph:

Indication 1) Traditional herbal medicinal product for the relief of minor inflammatory conditions of the skin.

Indication 2) Traditional herbal medicinal product used in excessive perspiration of hands and feet.

2.3. Specified strength / posology / route of administration / duration of use for relevant preparations and indications

Information on posology, route of administration, duration of use from products on the market:

Juglans regia leaves (comminuted herbal substance): 4 - 6g as a decoction in 200 ml of boiling water. Apply as a dressing to the affected areas of the skin three times daily. Not to be used for more than 1 week.

Information on posology, method of administration, duration of use from literature for relevant preparations

Commission E Monograph. *Juglans regia* L. (Bundesanzeiger No 101, published June 1, 1990).
Dosage: Unless otherwise prescribed: Topically for cataplasms and partial baths: 2 – 3 g dried leaf per 100 ml water or equivalent preparations.
Duration of use: no information

Deutcher Arzneimittel-Codex (2009)
Dosage: 2 3 g of the leaves / 100 ml of boiling water, heat for 15 min and then strain.
Topical application for cataplasms or rinses

Herbal Drugs and Phytopharmaceuticals *(*Wichtl*, 2004)*
Dosage: Place 1.5 g of finely cut dried leaf into cold water. Heat to boil and then strain after 3 – 5 min.
Oral route: drink one cup of tea 1 – 3 times daily.
Topical route: For cataplasms and rinses, decoct 5 g of dried leaf in 200 ml water. (1 teaspoon=about 0.9 g).
Duration of use: no information

*Herbal Medicinals, (*Miller & Murray*, 1998)*
Dosage: Topically. Place 5 g (5 teaspoons) of the chopped drug in 200 ml water.
Duration of use: no information

*Herbal Medicine, (*Weiss & Fintelman*, 2000)*
Daily dose: Oral route: add one tablespoonful of the finely chopped drug to ½ liter cold water, bring to a boil and strain after three to five minutes. Drink two to three cups each day.
Topical route: The decoction for compresses is made using around five tea spoonfuls of the drug.
Duration of use: no information

Lehrbuch der Biologischen Heilmittel (Madaus, 1938)
Dosage: orally: 2-3 spoonfuls (=3 – 4.5 g) of leaves in hot water (cup). Drink up to four times daily.
Bath: 0.5 – 1.0 kg of leaves, boil in a few liters of water and add to the morning and evening bath.
Maximal dose is not described.
Duration of use: no information

Medicinal Herbs: A Compendium (Gehrman et al. 2005)
Dosage: 2-3g (2 -3 teaspoons)/150 mL , cold maceration, heat to boiling point for 15 min, for poultice and as bath additive
Duration of use: no information

Medicinal Plants of the World, Wyk and Wink, (2004)
Dosage: An infusion is made by heating 1.5 g of the herb in a cup of water to boiling point.
For dressings or lotions, a decoction of 5 g in 200 ml water is recommended.

PDR for Herbal Medicines, Gruenwald et al.(ed.) (2000)
Dosage: Comminuted drug for decoctions for external use - soak 2 teaspoonfuls of drug in 1 cup of water, boil and strain.
An infusion is prepared by using 1.5 g of finely cut drug, soaked in cold water, brought to simmer and strained after 3 to 5 minutes.
The average daily dose for external use is 3 to 6 g of drug.
Duration of use: no information.

Ziołolecznictwo, Ożarowski, (1976)
Dosage: Place 20 – 30 g of the leaves/500 ml of hot water for mouthwash, compresses and cataplasms.
Duration of use: no information

On the basis of the information on traditional and current dosages, information on duration of use from clinical studies (see section 4.2.2.) and the requirements for specified strength and specified posology, the following is recommended for *Juglans regia* leaves included in the monograph:

Posology

Adults, elderly

Comminuted herbal substance as decoction

For cutaneous use: 4-6 g of dried leaves in 200 ml of boiling water as a decoction. Apply as a wet dressing to the affected areas of the skin three times daily.

There is no relevant use in children and adolescents under 18 years of age.

Duration of use

Duration of use is limited to one week due to possible presence of juglone.

3. Non-Clinical Data

3.1. Overview of available pharmacological data regarding the herbal substance(s), herbal preparation(s) and relevant constituents thereof

In vitro experiments

Antioxidant activity

Almeida et al. (2008) described scavenging effects of an ethanol:water (4:6) extract of *Juglandis regia* leaves. Scavenging activity was established on reactive oxygen species(ROS) [hydroxyl radical($HO^{\cdot}$), superoxide radical ($O^{\cdot}_2$), peroxyl radical ($ROO^{\cdot}$) and hydrogen peroxide (H_2O_2)] and reactive nitrogen species (RNS) [nitric oxide ($^{\cdot}NO$) and peroxynitrite anion ($ONOO^-$)]. The walnut leaves extract presented strong antioxidant effects against all studied reactive species with inhibitory concentrations (IC_{50}) in the range of the µg/ml level. Values of IC_{50} for the ROS $O^{\cdot}_2$ and H_2O_2 were 47.6 ± 4.6, 383 ± 17 µg/mL, respectively. The IC_{50}s for NO and $ONOO^-$ were 1.95 ± 0.29 and 1.66 ± 0.10 µg/mL, respectively. Value acquired for $ROO^{\cdot}$(the oxygen radical absorbance capacity) was 2.17±0.22 µmol Trolox equivalents/mg extract.

Authors suggest that observed antioxidant activity results partly from presence phenolics compounds in the extract (total phenolics content was 270 ± 3 mg of gallic acid equivalents/g of lyophilised extract).

Similar potent radical scavenging activity in the range of IC_{50} of 9.88 – 96.29 µM for six isolated phenolic compounds from the methanol extract of the leaves of *Juglans sinensis* were established by An et al. (2005).

Another study of radical scavenging activity of acetone, ethyl acetate and methanol extracts of several plants, *Juglans regia* leaves included was presented by Miliauskas et al. (2004). Dried material (6-10 g±0.01 g) and 100 ml of solvent was used. The extracts were filtered and concentrated at 40^0C. Radical scavenging activity was established with use of spectrophotometric method against stable 2,2-

diphenyl-2-picrylhydrazyl hydrate (DPPH)•. Methanol extract was the most effective DPPH radical scavenger with inhibition of 67.8±0.2 %, ethyl acetate and acetone extracts induced 33.9±2.3 and 25.3±1.6 % of inhibition, respectively.

Ethanolic extracts of several plants, *Juglans regia* leaves included, was tested for its antioxidant activity to inhibit of superoxide anion formation and lipid peroxidation levels *in vitro* by Çoban et al. (2003). The dried and chopped leaves of *Juglans regia* (20 g) were extracted with ethanol (75% aqueous, 300 ml) for 24 h by using Soxhlet apparatus. Inhibitory effects of the extract on superoxide formation and on lipid peroxidation are shown in the Table 1 and Table 2).

Table 1. Inhibitory effect of *Juglans regia* extract on superoxide anion formation

	Concentration (mg/ml)	Percent of control	IC_{50}
Juglans regia	0.5	85±1	1.39
	1	63±2	
	2.5	22±1	
	5	5±5	
	10	0±0	

Each value represents the mean±SD of 2-4 independent experiments

Ethyl alcohol only, control for the extract

Table 2. Inhibitory effect of *Juglans regia* extract on lipid peroxidation

	Concentration (mg/ml)	nMol MDA/g tissue	Percent of control	IC_{50}
Control		50.27±1.30	100	
Juglans regia	2.5	31.44±2.10	63	3.3
	5	15.56±1.30	31	
	10	9.15±0.11	18	

Each value represents the mean±SD of 2-4 independent experiments

Ethyl alcohol only, control for the extract

Antioxidant capacity of *Juglans regia* aqueous extract was established via haemolysis inhibition of human erythrocytes and bacteriophage P22 and Salmonella typhimurium model protection from the oxidant challenge by H_2O_2 (Giao et al. 2010). To prepare the aqueous extract 1 g of finely milled powdered dried leaves added to 110 ml of boiling water for 5 min and filtered through 0.45 µm filters and lyophilized. For the haemolysis and the phage method extracts were reconstituted to obtain 0.0025% (w/v), or at ca. 0.02% (w/v). In result, the assays showed positive antioxidant effects for the xtract of *Juglans regia* leaves in haemolysis test, but no effect in bacteriophage test.

Antibacterial activity

Çitoğlu and Altanlar (2003) tested antibacterial and antifungal activity of several plants, *Juglans regia* included. The dried and chopped samples of 20g of *Juglans regia* leaves were extracted with ethanol (75% aqueous, 150 ml) for 24 hours by using a Soxhlet apparatus. The extract was tested

with the disc diffusion method against gram-negative (*Escherichia coli, Pseudomonas aeruginosa*) gram-positive (*Staphylococcus aureus, Bacillus subtilis*) and fungi (*Candida albicans,Candida galabrata*, *Candida krusei*) antimicrobials. The antimicrobial effects were compared with activity of the antibiotic ampicillin, and the antifungal agent fluconazole and positive control (ethanol) (Table 3).

Table 3. The inhibition zones diameter of the aqueous extract of *Juglans regia*.

Diameters of the inhibition zones (mm)							
	E. coli	P.aeruginosa	B. subtilis	S. aureus	C.albicans	C.galabrata	C. krusei
Juglans regia	10	10	-	12	11	8	8
Ampicillin 25 ng	12	N.T.	13	15	N.T.	N.T.	N.T.
Fluconazole 25 ng	N.T.	N.T.	N.T.	N.T.	18	20	20
Control (Ethanol)	_	_	_	_	_	_	_

N.T. : not tested

(-): no inhibition zone

Results of the test show good antimicrobial activity of the aqueous *Juglans regia* extract.

Kong et al. (2008) reported, that juglone displays different inhibiting activity against 3 main enzymes from *H pylori*: cystathionine g-synthase (HpCGS), malonyl-CoA:acyl carrier protein transacylase (HpFabD), and b hydroxyacyl -ACP dehydratase (HpFabZ). Inhibition of HpCGS was non-competitive with IC_{50} value: 7.0±0.7 μmol/L; inhibition of HpFabZ was uncompetitive: IC_{50} = 20±1 μmol/L and inhibition of HpFabZ was competitive: IC_{50}=30±4 μmol/L. Authors conclude, that as juglone acts as multitargeted inhibitor it may be an encouraging agent against of bacterial resistance development.

Antibacterial activity of methanolic and aqueous *Juglans regia* extracts was decribed by Mehrabian et al. (2000) against Gram +, airborne *Bacillus cereus* and *Bacillus mycoides*. Fifty g of powdered plant was added to 100 ml of each solvent: water or methanol. After remaining in room temperature for 24 hours the extracts were purified by distillation. The plates with cultured bacteria were incubated at 37^{o}C for 24 hours and 1ml of 1:10 or 1:20 dilution of the aqueous and methanolic extracts were added respectively do the cultured plates. Indication of the antibacterial activity was estimated after 48-72 hours.

Darmani et al. (2006) examined the activity of the aqueous extract of *Juglans regia* on proliferation of Balb/C 3T3 mouse fibroblasts and viability of cariogenic bacteria (*Streptococcus mutans, Streptococcus salivarius, Lactobacillus casei and Actinomyces viscosus*). The results showed the significant increase in cell proliferation by 255% ($p<0.0001$). The walnut extract exhibited antibacterial effect against cariogenic bacteria: the most sensitive were: *A. viscosus*, followed by *S. mutans, S. salivarius*, with *L. casei* being the most resistant.

Pereira et al. (2007) investigated leaves of different 6 cultivars of *Juglans regia* for its antioxidant and antimicrobial activity. After determination of the content phenolic compounds (naphtoquinones and flavonoids), the antimicrobial activity was screened on Gram positive (Bacillus cereus, B. subtilis, *Staphylococcus aureus*) and Gram negative (*Pseudomonas aeruginosa, Escherichia coli, Klebsiella penumoniae*) bacteria and fungi (*Candida albicans, Cryptococcus neoformans*). Juglone was not found in the samples (due to low solubility in water and volatile in steam). Three powdered subsamples (~ 5 g) were extracted with 250 mL of boiling water for 45 min and filtered, frozen, lyophilized and redissolved in water at concentrations of 100 mg/mL and 10 mg/mL.

All tested cultivars showed antimicrobial activity, Gram positive (*Bacillus cereus*) being the most susceptible (MIC 0.1 mg/mL). The Lara cultivar walnut leaves were most active, therefore its extract was tested against 18 Staphylococcus sp. strains isolated from sputum, pus and blood. The most

susceptible were the strains isolated from sputum (MIC's range of 0.1 mg/mL), while MIC's for the strains isolated from pus and blood ranged between 0.1 and 1 mg/mL.

Lara cultivar exhibited as well highest antioxidant activity in three tests: reducing power assay (EC_{50}=192 mg/mL), scavenging effects on 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radicals (EC_{50}=0.151 mg/mL) and β-carotene linoleate bleaching model (EC_{50}=0.742 mg/mL). These results were much better than those acquired for control standard substances – α-tocopherol and 2-tert-butyl-4-methoxyphenol (BHA). In conclusion authors suggest therapeutic use of walnut leaves extract against human gastrointestinal and respiratory infections.

Qa'dan et al. (2005) tested antimicrobial activity of *Juglans regia* extract against acne developing bacteria: *Propionibacterium acne, Staphylococcus aureus* and *Staphylococcus epidermidis*. Walnut leaves (500 g) were air dried and extracted with acetone: H_2O_2 (7:3.5 L). The extracted material was evaporated to 1.5 L , filtered and defatted. Later was shaken with ethyl acetate and the water residue (46 g) was subjected to column chromatography with MeOH-H_2O 1:1 (12 L). The tested extract was eluted with acetonH_2O 7:3 (5 L) and frieze dried (7. g). Antibacterial activity of the extract was determined by disk diffusion method. Zones of inhibition were significantly higher than control standard tea tree oil effects and ranged from 15.8 to 17.6 mm against *Propionibacterium acne*, 11.3-15.7 mm against *Staphylococcus aureus* and 12.9 – 15.5 mm against *Staphylococcus epidermidis*. However were minor compared to the effects received from doxycycline or clindamycin. Autors confirm beneficial role of *Juglans regia* in acne treatment.

Cruz-Vega et al. (2008) tested in vitro by a microplate dilution assay antimycobacterial effects of various Mexican plants against *Mycobacterium tuberculosis* strain H37Rv. Hexane, methanol, ethanol and aqueous extracts of *Juglans regia* leaves were used. For hexane and methanol extracts, 60 g of leaves were extracted (8.5%w/v), for ethanol – 60 g of the material was macerated with ethanol (10%w/v) at room temperature, the aqueous extract was obtained by decoction of 30 g of material (6%w/v) and then frieze-dried. Hexane, methanol and ethanol extracts were concentrated under vacuum to yield dry residues. All extracts were stored in the dark at 4^0C until use. The results are shown in Table 4.

Table 4. Antimycobacterial activity of *Juglans regia* extracts

	Extract tested	**Yield (%)**	**Minimal inhibitory concentration (MIC) µg/ml**
Juglans regia leaves	Hexane	5.0	NA
	Methanol	6.6	125
	Ethanol	15.0	NA
	Aqueous	13.3	NA

All biological assays were done at least by duplicate

NA _ they did not present activity at 1000 µg/ml

In conclusion, the leaves extracts did not have evident antibacterial activity. As the MIC value for the methanol extract was ≥ 100 µg/ml, the extract was considered inactive against *M. tuberculosis* (Cos et al. 2006).

Antilisterial effects of *Juglans regia* were evaluated by the agar diffusion and the macrodilution method (Altanlar et al. 2006). The leaves of *Juglans regia* (20 g) were extracted with ethanol (75% aqueous, 150 ml) for 24 h by using Soxhlet apparatus.

The tests were performed against *L. monocytogenes, L. ivanovii, L. murrayi* and *L. inooocua*. Minimum inhibitory concentrations (MIC) of evaluated *Juglans regia* extract were 12.5 µg/ml against *L. monocytogenes* and *L. murrayi*, 25 µg/ml against *L. ivanovii* and without any effect against

L. inooocua. MIC's of the active control ofloxacin were: 3.125µg/ml against L monocytogenes and 6.25 µg/ml against other species.

Antiviral activity

Min et al. (2002) established antiviral activity of quinones, juglone included, on RNase H activity associated with HIV-1 reverse transcriptase. The effect of juglone was moderate and percentage of inhibition after applying the concentration of 100 µM of juglone was 51.4±1.0 (in triplicate) with value of IC_{50} =95 µM.

However strong inhibitory effects of juglone was seen on RNA-dependent polymerase (% inhibition=95.5±0.5 with IC_{50} =8 µM), on DNA-dependent polymerase (% inhibition=99.9±0.0 with IC_{50} =5 µM) and on DNA polymerase I activitity (% inhibition=70.3±1.7 with IC_{50} =10 µM).

Mouhajir et al.(2001) investigated antiviral activity of methanol extracts of 75 Moroccan plants, *Juglans regia* included. The extracts were prepared with use of a 100-200 g of the dried plant material soaked in 100 – 200 ml of methanol, filtrated and washed again with another 200 ml aliquot of methanol. The filtrates were evaporated to dryness and finally diluted at concentration of 2 mg/ml. Extracts were evaluated against: herpes simplex virus (HSV), Sindbis virus (SINV) and poliovirus (polio) at non-cytotoxic concentrations. Viruses tested represent models for skin infections (HSV), gastro intestinal infections (polio) and mosquito borne infections (SINV).

The results of experiment are shown in the Table 5.

Table 5. Antiviral activity of *Juglans regia* methanol extract (MIC µg/mL) (Mouhajir et al. 2001).

	SINV		**HSV**		**Polio**	
Juglans regia	Light	Dark	Light	Dark	Light	Dark
	1.5	12.5	1.5	1.5	25	25

Light and dark activities

Antifungal activity

Antifungal activity of dried aqueous extracts of 22 medicinal plants, *Juglans regia* leaves included (100:1) was tested against fungal isolates in culture*: Microsporum canis* (S14, S20, and SH41), *Trichophyton mentagrophytes* (SH13, SH1, SH8), and *Trichophyton violaceum* (S5, SH32, SH38). Fungi were isolated from patients with tinea capitis. As reference antibiotic griseofulvin was used. Results are presented in Table 6. (Ali-Shtayeh and Abu Ghdeib 1999).

Table 6. Mycelial inhibition of tinea capitis dermatophytes (Ali-Shtayeh and Abu Ghdeib 1999).

Means* of percentage mycelial inhibition of tinea capitis dermatophytes				
Extract (15 µ/ml medium)	Dermatophytes			
	M. canis	T. mentagrophytes	T. violaceum	Total
Juglans regia	100.0±0.00	97.6±2.42	100.0±0.00	99.2±0.8
**Griseofulvin	100.0±0.00	100.0±0.00	100.0±0.00	100.0±0.00

*Means of three replicate plates for each of three isolates for each species.

**Concentrations of reference antibiotic (MIC) was 0.6 µg/ml for M. canis, 2.5 µg/ml for T. mentagrophytes, and 0.6 µg/ml for T. violaceum.

The MIC values of *Juglans regia* extract ((15 µ/ml medium) showed high: 97.6 to 100 % inhibition of mycelial growth and were comparable with griseofulvin activity. However, according to other experiments *Juglans regia* was inactive against *Candida albicans*.

Anticancer activity

In vitro experiments

Antioxidant and antiproliferative activities of the methanolic and petroleum ether extracts of *Juglans regia* leaves were decribed by Carvalho et al. (2010). Freeze-dried plant material (1.5 g)was mixed with methanol or petroleum ether (3 x 25 ml). The extracts were filtered and concentrated to dryness under reduced pressure (40^0C). The methanolic and petroleum ether extraction efficiency in relation to dry matter was 27.7% and 1.1%, respectively. In vitro performed tests showed also significantly different phenolic content: 94.39±5.6 and 27.92±1.4(mg GAE/g extract), EC_{50} values for the DPPH free radical scavenging capacity: 0.199±0.023 and 2.921±0.740 (mg/mL) ant IC_{50} values for the antihemolytic activity: 0.06±0.005 (mg/mL) and no activity respectively.

Antiproliferative activity (IC_{50} , mg of methanolic extract/mL)of the *Juglans regia* leaves towards cultures A-498, 769-P and Caco-2 cells were: 0.226±0.053; 0.352±0.055 and 0.229±0.014, respectively. Values were presented as mean±SEM of three independent experiments, performed in triplicate. Presented results exhibit the effective antihemolytic and human renal cancer antiproliferative effects.

Enzyme activity inhibition

Muto et al. (1987) described inhibition of microsomal cytochrome P450 monooxygenase activity in and rabbit liver and in human placenta. Strong inhibition of synthesis of placental estrogen was seen. Authors explain the inhibition by direct interaction of naphtoquinone with the heme

Significant inhibition by juglone of urease activity, enzyme responsible for catalyzing the hydrolysis of urea has been found by Kot et al. (2010). Supposed arylation of urease thiol groups by juglone is responsible for this effect.

Antityrosinase activity of methanol extracts of *Castanea sativa* stem bark, *Eucalyptus camaldulensis leaves* and *Juglans regia* leaves tested Özer et al. (2007). Tyrosinase inhibition play important role in treatment uneven skin hyperpigmentation. Plant material (100 g) was extracted with 80% methanol, filtrated and evaporated to dryness under vacuum. The quantity of ellagic acid in the Juglandis folium extract was the highest: 16.25±0.512 % (n=6±SD). Walnut extract manifested as well the highest antityrosinase activity with IC_{50} = 505 µg/mL, which was found to be 1.2 and 1.8 fold higher than that of *Eucalyptus camaldulensis* leaves and *Castanea sativa* stem bark, respectively. The Author concluded, that the extracts in gel formulations containing *Juglans regia* could be used as skin-whitening agents.

Vasorelaxant Activity

In the study of Perusquia et al. (1995) the aqueous extract of *Juglans regia* leaves inhibited in a concentration dependent manner (0.5-12 mg/ml) the maximal contractile response induced by noradrenaline in isolated rat thoracic aorta.

In vivo experiments

Antidiabetic activity

Mohammadi et al (2011) with in a model of streptozocin diabetes in rats reported significant antidiabetic activity of the aqueous extracts of *Juglans regia* leaves in rats. The extracts were prepared from powdered dry leaves (700 g), which were subjected twice for 24 h to extraction with 90% ethanol in room temperature. The filtrate was evaporated until dry to 80.4 g of powder, which was later diluted in water to receive doses of 200 and 400 mg/kg. Diabetes was induced in rats by single injection of 55 mg/kg i.p. of streptozocin (STZ) and identified in animals with glucose level over 300 mg/dL. Four groups of 8 rats were used in experiment. After ten days diabetic groups III and IV received orally 200 and 400 mg/kg of the extract of *Juglans regia* leaves for 28 days. Control (I) group received buffer i.p. After autopsy glycosylated hemoglobin, triglyceride, cholesterol, LDL, VLDL and HDL levels were determined (Table 7.)

Table 7. The effect of the extract of the *Juglans regia* leaves on glycosylated hemoglobin, triglyceride, cholesterol, LDL, VLDL and HDL levels (Mohammadi et al. 2011).

Group **Parameters**	**Control** **I**	**Diabetic** **II**	**STZ + 200** **III**	**STZ + 400** **IV**	**F value** **df (3, 20)**
Glycosylated Hemoglobin (%Hb)	6.52 ± 0.12^{a}	12.33 ± 0.29^{c}	9.20 ± 1.11^{b}	6.41 ± 1.05^{a}	Sig 12.43
Triglycerides mg/dL	$64.1 \pm 4.72^{a,b}$	85.2 ± 3.58^{c}	69.18 ± 3.52^{b}	57.1 ± 2.4^{a}	Sig 11.78
Cholesterol mg/dL	74.5 ± 3.66^{b}	84.1 ± 4.61^{c}	69.7 ± 3.77^{b}	56.9 ± 5.19^{a}	Sig 15.88
LDL mg/dL	33.22 ± 3.1^{b}	41.5 ± 2.6^{c}	$22.95 \pm 3.6^{a,b}$	19.1 ± 3.3^{a}	Sig 7.82
VLDL mg/dL	$11.74 \pm 1.49^{a,b}$	16.32 ± 1.45^{c}	13.54 ± 1.96^{b}	11.42 ± 0.98^{a}	Sig 13.39
HDL mg/dL	$31.14 \pm 1.49^{a,b}$	27.2 ± 0.55^{a}	$29.76 \pm 1.33^{a,b}$	31.52 ± 2.71^{b}	NS 4.87

The values are mean ± SE for 8 rats in each group. Means with different superscript (a, b, c, d) within a column are significantly different from each other at $P<0.05$ as determined by Duncan's multiple range test.

Glucose concentration in blood of the II and IV group was significantly diminished. The extract of *Juglans regia* leaves at the dose of 400 mg/kg after 21 of injections restored the glucose levels almost to the control values. Also insulin levels in group III and IV 9400 mg/kg) reached almost of those in the control (I) group.

Similar results were presented by Asgary et al. (2008) with an ethanolic extract of *Juglans regia* leaves in the alloxan-induced diabetes in rats. There was no difference between *Juglans regia* extract (200 mg/kg) when compared with antidiabetic activity of glibenclamide 0.6 mg/kg. Both treatments tended to bring the levels of glucose, insulin and glycosylated hemoglobin to near normal. Histological observations showed that the size of Langerhans islets enlarged significantly as compared to diabetic control rats with no treatment.

Similar antidiabetic action of *Juglans regia* leaves extracts were also described by Gholamreza an Hossein (2008) and Dzhafarova et al. (2009) in Wistar rats with experimental diabetes.

Anticancer activity

Inhibitory effect of juglone on azoxymethane-induced intestinal carcinogenesis was presented by Sugie et al. (1998). At the start of experiment male F344 rats 5 weeks of age received orally in a diet 200 ppm of juglone, control group received only diet. Some groups of rats were receiving once a week for 3 weeks s.c. injections of azoxymetane (AOM, 15 mg/kg bw) to induce tumorigenesis in control rats or together with juglone. After autopsy the number and size of tumors were measured. Juglone treatment decreased the incidence (% animals with tumors) and multiplicity (number of tumors/animals) in small (and entire intestine compared to the group with AOM alone ($p<0.05$ in each). The maximum diameter of tumors was smaller than that of rats given AOM alone ($p<0.05$).

Insect growth regulatory activity

In order to develop of alternate strategies for protection of important crops, juglone was found to induce lethal effects on the growth of insect red cotton bug (Dysdercus koenigii Fabr.) in concentrations of 1-10 µg/nymph (Banerjee et al. 2001; Magdum et al. 2001).

Insecticidal activity

Experiments in vitro were performed with the aqueous extract of several plants, *Juglans regia* included against larvae of Trichoferus griseus (Fabricius 1792) living on Pinus brutia L (Civelek and Colak 2008). Insecticidal effects of *Juglans regia* extract were compared with insecticide Bensultap and synthetic chemical preservation (chromated copper arsenate, Tanalith C). Extract was prepared from 2 g plant sample and extracted with 100 ml of water using a Soxhlet hot water extractor. Afterwards extract was stored at 5^0C until use. The average number of alive larvae (± SE) at the end of the experiment were: 0.4±0.05 ($p<0.05$) after Juglans regia extract, 0.0±0 after Bensultap – a positive control, and 0.666±0.059$p<0.05$) after Tanalith C.

3.2. Overview of available pharmacokinetic data regarding the herbal substance(s), herbal preparation(s) and relevant constituents thereof

No data are available concerning *Juglans regia* leaves pharmacokinetics due to its complex phytochemical composition.

Juglone pharmacokinetics

Juglone (5-hydroxy-1,4-naphtoquinone) is present in roots, nut hulls, bark, wood and leaves of the European walnut (*Juglans regia*). It is reactive compound due to its redox activity and elctrophilicity. Metabolic reduction of juglone involves generation of hydroquinone and further a enzyme catalysed conjugation of the hydroxyl groups with glucuronide or sulfate. The glucuronides and sulfate coniugates formation as reduced species effect produce detoxification of quinone. In the study of Chen et al. (2005) in male F344 rats the disposition of 14C-labelled juglone (0.1-10 mg/kg) administered by oral, intravenous and dermal (topical) route was determined (Table 8.).

Table 8. Disposition of juglone-derived radioactivity (% of dose ± SD) in male F344 rats (n=4) 24 or 72 h following administration. (Chen et al. 2005)

	0.1 mg kg^{-1}, oral, 24h	**1 mg kg^{-1}, oral, 24h**	**1 mg kg^{-1}, oral, 72h**	**10 mg kg^{-1}, oral, 24h**	**0.1 mg kg^{-1}, i.v., 24h**	**4 mg kg^{-1}, dermal, 24h**	**4 mg kg^{-1}, dermal, 72h**
Urine	18.2 ± 3.6	21.3 ± 1.6	23.6 ± 2.8	50.0 ± 7.2[a]	32.8 ± 2.5	2.5 ± 0.7	6.3 ± 0.6

	0.1 mg kg^{-1}, oral, 24h	**1 mg kg^{-1}, oral, 24h**	**1 mg kg^{-1}, oral, 72h**	**10 mg kg^{-1}, oral, 24h**	**0.1 mg kg^{-1}, i.v., 24h**	**4 mg kg^{-1}, dermal, 24h**	**4 mg kg^{-1}, dermal, 72h**
Faeces	63.2 ± 6.2	65.0 ± 3.6	69.2 ± 3.5	41.5 ± 7.3[a]	23.3 ± 2.0	1.3 ± 0.4	5.3 ± 0.4
Tissues	1.1 ± 0.2	1.6 ± 0.1	0.7 ± 0.1	3.0 ± 0.9	28.6 ± 3.9	6.4 ± 1.9	4.8 ± 0.4
Intestinal contents		5.7 ± 1.6	0.1 ± 0.1	3.8 ± 0.9	3.6 ± 0.8	0.6 ± 0.1	1.2 ± 1.0
$C0_2$ and volatiles	n.d.[b]	n.d.	n.d.	0.1 ± 0.1	n.d.	0.1 ± 0.1	n.d.
Unabsorbed	-	-		-		80.2 ± 6.5	38.7 ± 2.0
Skin at the dosing site	-	-	-	-	-	2.9 ± 0.4	8.4 ± 2.1
Total	89.2 ± 2.1	93.6 ± 3.5	93.6 ± 2.1	98.3 ± 2.0	88.4 ± 2.4	96.0 ± 8.4	67.2 ± 2.1

[a]Faecal contamination of the urine made the distribution between urine and faeces uncertain
[b]Not determined

The study shows that less than 50% of an oral dose (0.1 to 10 mg/kg) and less than 20% of a topical application on skin (4 mg/kg) were absorbed. After absorption, juglone radioactivity was almost completely excreted in faeces and urine in 24 h after oral administration, with only 1-3% left in the body. Comparatively highly concentration of juglone was present in kidney, regardless of the route of application. Five metabolites as a result of two electron reduction, conjugated later by glucuronide and sulfate, were identified in urine of rats after oral administration of juglone.

Overview of pharmacokinetics

Pharmacokinetics of 3H-juglone (0.02 mg/kg, i.v.) was studied by Aithal et al. (2011) in C57/BL mice. After the bolus dose about 35% of juglone accumulated within 15 min in the kidneys, with the a half-life of about 2 hours.

There is no data on juglone pharmacokinetics in humans.

Labelled [14C]hydroquinone pharmacokinetics was studied after topical application of the cream containing 2%hydroquinone to the skin of human volunteers (Wester et al., 1998). After 24 h the 45% bioavailability was found with a rapid and contiinuous movement of the hydroquinone to the stratum corneum. The maximal plasma concentrations were found at about 4 h after application and most radioactivity was eliminated with urine within 24h as glucuronides.

Due to lack of data on pharmacokinetics of walnut leaves no conclusions can be drawn.

3.3. Overview of available toxicological data regarding the herbal substance(s)/herbal preparation(s) and constituents thereof

Walnut has been reported allelopathic, to actively limit the growth the other various plant species, including herbs, shrubs, deciduous trees nearby (von Kiparsky et al. 2007; Weir et al. 2004). In Turkey, the practice of intercropping with the use of *Juglans regia* is popular (Ercisli et al. 2005). It is reported, that wood shavings or heartwood of black walnut (*Juglans nigra*) causes laminitis in horses, but such effects of *Juglans regia* has not been determined (Harman 2002).

Juglone has possible potential for human exposure as a result of appearance in hair dye formulations and use in dietary supplements.

Juglone is toxic to mammalian cells *in vitro* and also to small animals in *vivo* (Soderquist 1973). Juglone is toxic to fish at <0.1 mg/L and can kill the fish before degrading in the natural environment (Marking 1970).

It is also very toxic to insects as Drosophila larvae. In adult males which are exposed to a 10% aqueous sucrose solution of juglone a strong mutagenic response was found, mainly in first generation. The size of the brood was significantly diminished (Clark 1982; Harada www.agnet.org/library/bc/45016/)

To determine the amount of juglone in herbal preparations the content in the decoction/infusion of *Juglans regia* leaves by GC/MS analysis was analyzed by Matławska et al. (2012a, 2012b).The infusion contained detectable juglone amounts ≅ 0.1%, but the amount of juglone in the decoction was found below the detection limit (<0,0003%). Simulation of the conditions found in the lumen of the stomach and on the human skin surface at body temperature of 37C, induced by the change of pH of the tested solutions to 2.0 or 4.0 values, does not increase the concentration of juglone (<0,0003%). This means that juglone is not present in the acidic environment of the skin and in the gastric juice after topical or even oral ingestion. The authors have demonstrated that decoction of walnut leaves, is the most appropriate for safe use for topical application on to the skin, due to a very low juglone content.

Acute toxicity

Acute toxicity of juglone was studied by Aithal et al (2011). It was found that oral LD_{50} for rat is 112 mg/kg, and for the mouse 2.5 mg/kg. Repeated dosing produced accumulation of juglone adducts in the kidney. After 7-day treatment of 1mg/kg juglone of mice, the histological changes were seen in the kidneys, but not in liver, spleen and heart (Aithal et al. 2011).

Ichthyotoxic activity.

Old tradition of use ichthyotoxic plants in Europe is confirmed by ethnobotanical records as early as in Spain in 1255 where formally made it public and forbade their use.

In folk tradition *Juglans regia* is one of the 32 plants registered in common use, but this kind of practice is currently curtailed (Alvarez Arias 2000).

Prooxidant activity

Juglone acts as a generator of superoxide anion radicals. Cytotoxic activity of juglone depends on the decrease in glutathione due to enzymatic redox cycling of naphtoquinones and production of DNA and protein adducts as was elucidated by Ross et al. (1986), Öllinger and Brunmark (1991) and O'Brien (1991). Similar effects were obtained by Murakami et al. (2010), where juglone markedly stimulated the lipid peroxydation. Juglone also produced 8-hydroxy-2'-deoxyguanozine (8-OHdg), the base adduct in DNA. Moreover the juglone formed semiquinone radical in the presence of ferrous ion.

Cytotoxicity of juglone

Juglone induced cell death by apoptosis and necrosis through diverse mechanisms such as induction of oxidative stress, cell membrane damage and clastogenic effect (Aithal et al. 2009; Babula et al. 2009; Bayer et al. 2005; Inbaraj and Chignell 2004; Öllinger and Brunmark 1991).

Cytotoxic activity of juglone was demonstrated after 72 h of incubation against leukemia (HL-60), melanoma (MDA-MB435), brain (SF-295) and colon (HCT-8) human cancer cell lines and against peripheral blood mononuclear cells (PMBC) as the control normal cell lines. The cytotoxicity of all

compounds was tested using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2Htetrazolium bromide (MTT). Juglone was dissolved in DMSO 1% and was added to each well and incubated for 72 hours.

Control groups received DMSO only. The supernatant was replaced by fresh medium containing MTT (0.5 mg/mL) and after 3 h the MTT formazan product absorbance was measured. Doxorubicin (Dox) was used as positive control. The MTT analysis showed that the IC50 juglone values ranged from 7.6 to over 28.7 µM between cells after 72 h of incubation. IC50 ranged in HCT-8 and HL-60 and/or MDA-MB435, respectively. For normal PBMC, IC50 values for Juglone were over 28.7 (Table 9. Montenegro et al. 2010)

Table 9. Cytotoxic activity of juglone and its derivatives on tumor cell lines, on peripheral blood mononuclear cells (Montenegro et al. 2010)

	HL-60	**MDAMB 435**	**SF-295**	**HCT-8**	**PBMC**
Juglone	>5 (28.7)	5 (28.7)	2.97 (17.0) 2.01–4.38	1.33 (7.6) 0.74–2.39	5 (28.7)
Dox	0.02 (0.03) 0.01–0.02	0.48 (0.8) 0.34–0.66	0.23 (0.4) 0.19–0.25	0.01(0.02) 0.01–0.02	0.97 (1.7) 0.52–1.80

*Data are presented as IC50 values and 95% of confidence interval for leukaemia (HL-60), melanoma (MDAMB-435), nervous system (SF-295), colon (HCT-8) cancer cells and peripheral blood mononuclear cells (PBMC). Doxorubicin (Dox) was used as a positive control. Experiments were performed in triplicate.

In other similar study cytotoxic and genotoxic effects of juglone were demonstrated against melanoma B16F1 cell line.

Contrary effects were described by Monks et al. (1990) in the study of induction by quinones (juglone) epidermal ornithine decarboxylase (ODC) and promotion skin tumors growth in mice. Single topical application of juglone produced dose-dependent increase in epidermal ODC activity in the dose range of 880-3250 nmol/mouse. It also induced promotion of papilloma formation (1760nmol) with the highest response determined with a three times/week topical application. Moreover, juglone lightly converted papillomas to carcinomas (carcinoma/papilloma ratio of 0.35)

It was found that juglone inhibits several enzymes activity, including the members of the parvulin PPIase family (Pin1) by modifying sulfhydryl groups (Hennig at al 1998; Maruyama and Furutani 2000). Pin1 plays an important role in oncogenesis and its expression is increased in many human cancers.

Juglone, independently on Pin1 irreversible inhibition, can inhibit post-mitotic dephosphorylation and the exit of mitosis. Juglone affected cells had disturbed microtubule dynamics, showed loss of tubulin and formation of tubulin aggregates (Fila et al. 2008).

Juglone acts as an active inhibitor of RNA processing by blocking transcription of RNA polymerase II (Chao et al 2001). Moreover, juglone can induce apoptosis and/or necrosis probably by subsequent activation of caspases as other naphtoquinone - benzobijuglone from *Juglans mandshurica* (Li et al. 2007).

Juglone treatment inhibited intracellular mammalian protein thioredoxin reductase (TrxR) causing formation selenium-compromised forms of TrxR (SecTRAPs). TrxR-derived proteins action involves induction of caspase activation that cannot be prevented by Bcl2. SecTRAPs are devoid of thioredoxin reductase activity but can induce rapid cell death in cultured human A549 and HeLa cancer cell lines (Anestal et al. 2008).

In colonies of human cancer cells juglone induced apoptosis: it was found strongly cytotoxic and induced caspase-3/7 activation in HeLa cells, moreover induced apoptosis in Bcl2 overexpressing HeLa cells and in A549 cells (Cenas et al. 2006). .

In other model of cytotoxicity juglone inhibited growth and induced apoptosis in human gastric cancer SGC-7901 cells. It was shown that in the range of 5μM to 20 μM juglone activated caspase 3 in a concentration-dependent manner, Bcl-2 protein expression was down-regulated, level of the ROS generation was increased and up-regulation of Bax protein expression was seen.

The cytotoxic mechanism is induced by the activation of the mitochondrial death pathway with apoptosis and necrosis of gastric cancer cells Table 10. (Ji et al. 2009).

Table 10. Effect of juglone on the cell apoptosis of SGC-7901 cells (24h) (mean±SD, n=3) (Ji et al. 2009).

Group	Dose (μM)	Apoptosis rate (%)	Normal rate (%)	Necrosis rate (%)
Control	-	0.17 ± 0.06	95.13 ± 1.23	0.13 ± 0.06
Juglone	5	7.27 ± 0.15**	82.90 ± 0.90**	2.3 ± 0.36**
	10	10.47 ± 0.15**	83.73 ± 0.35**	5.13 ± 0.32**
	15	16.13 ± 0.25**	75.33 ± 0.25**	5.97 ± 0.25**
	20	27.60 ± 0.70**	63.07 ± 0.77**	7.27 ± 0.15**
HCPT	60	32.83 ± 0.35**	55.43 ± 0.67**	5.80 ± 0.30**

*Compared with control P<0.05.

**Compared with control P<0.01.

HCPT (Hydroxycamptothecine) – active control

Genotoxicity of juglone

No published data could be found on the genotoxicity of European walnut leaves.

Evaluation of genotoxicity of the *Juglans regia* extracts showed significant damage in the comet assay in concentrations above 250 ppm and more but not in the VITOTOX test. The extracts in the Ames assay showed potent antimutagenic effects against the direct acting mutagens NPD, sodium azide, and the S-9dependent mutagen 2-AF (Arora et all. 2005). The authors conclude, that plant extract can be mutagenic as well as antimutagenic depending on the test system used.

Juglone tested with *S. typhimurium* in the presence of S-9 mix was found positive in strain TA2637 and negative in TA98 and TA100 (Tikkanen et al. 1983; Edenharder and Tang 1997)

Tests with *S. typhimurium* without S-9 mix have given negative results: strains TA98, TA100, TA2637 (Tikkanen et al. 1983; Edenharder and Tang 1997; Juglone NTP http://ntp.niehs.nih.gov/go/19336).

A positive value of genotoxic activity of juglone in the two tests of *w/w* SMART asay of *Drosophila melanogaster* (498.0% of mosaic eyes induced per mM of compound at the lowest effective dose)was presented by Gaivao et al. (1999). The Ames test data for the other naphtoquinones were variablewith some positive results.

In conclusion the genotoxicity of juglone is scarce and weak.

3.4. Overall conclusions on non-clinical data

It can be concluded that despite that juglone as a quinone compound has been linked to mutagenic action, it is unstable and in dry leaves is present only in traces. On the basis of existing findings and

comparisons with other quinones, genotoxicity seems not truly evaluated and may be limited and weak and probably meaningless to humans following exposure to small amount of juglone.

Experimental preclinical data show antioxidant, antiviral, antibacterial, antifungal, antidiabetic and anti-inflammatory activity. Results of antimicrobial evaluation *in vitro* activity of *Juglans regia* extracts support the traditional use of European walnut.

The published data on pharmacological activities support the traditional use of preparations containing *Juglans regia* in the proposed indications.

4. Clinical Data

4.1. Clinical Pharmacology

4.1.1. Overview of pharmacodynamic data regarding the herbal substance(s)/preparation(s) including data on relevant constituents

There are no data on human pharmacodynamics.

4.1.2. Overview of pharmacokinetic data regarding the herbal substance(s)/preparation(s) including data on relevant constituents

There are no data on human pharmacokinetics.

4.2. Clinical Efficacy

4.2.1. Dose response studies

There are no specific data available on dose-response studies.

4.2.2. Clinical studies (case studies and clinical trials)

None were published on mono-preparations of European walnut.

4.2.3. Clinical studies in special populations (e.g. elderly and children)

No information available.

4.3. Overall conclusions on clinical pharmacology and efficacy

There are no data available from controlled clinical studies, therefore the medicinal use of *Juglans regia* leaves has to be regarded as traditional.

5. Clinical Safety/Pharmacovigilance

There are no adverse effects reported from the Member States, however allergic reactions to *Juglandacae* family should be considered.

5.1. Overview of toxicological/safety data from clinical trials in humans

There are no data from clinical trials available.

5.2. Patient exposure

None reported.

5.3. Adverse events and serious adverse events and deaths

Allergy

Allergic reactions following contact with walnuts are common. Both tree and its fruit can be responsible. Moreover cases of allergic contact dermatitis to walnut wood have been described. The history of 14 month of contact dermatitis with hand eczema, were seen in 35 year-old baker making walnut cakes. Prick testing confirmed flare reactions to walnut (Mendonca et al. 2005).

Pigmentation

The intense pigmentation and acute irritant contact dermatitis with large blisters involving the palms and fingers due to the contact with *Juglans regia* juice was described in Southern Italy (Bonamonte et al. 2001). After 5 days treatment with compresses of aluminium acetate solution the irritant contact dermatitis symptoms had declined, however pigmentation lasted 3 weeks.

Two cases of pigmentation and acute irritant contact dermatitis were described by Neri et al (2006) in children playing with green walnut husks.

Ethnic customs using of *Juglans regia* preparations as a tooth cleansing method, can induce pigmentation in the oral cavity (Ashri and Gazi 1990; Eisen 2000).

5.4. Laboratory findings

No data available.

5.5. Safety in special populations and situations

There are no reports of use *Juglans regia* leaves in children. The use of walnut leaves is not recommended in children younger than 18 years of age.

Drug interactions

None reported for *Juglans regia* leaves preparations. However, it is reported in literature that *Juglans regia* due to high tannin content may cause alkaloids to become insoluble and precipitate, as well as reduce the absorption of other drugs (Herr 2005).

Some authors mention that tannins may also interfere with iron absorption.

Use in pregnancy and lactation.

The walnut leaves bark should not be used during pregnancy and lactation.

Overdose

None reported.

Effect on ability to drive or operate machinery or impairment of mental ability

None reported

5.6. Overall conclusions on clinical safety

There are no reports of adverse effects of walnut leaves from Member States.

The allergic reactions in patients allergic to Juglandaceae should be considered.

6. Overall conclusions

The available data are sufficient to include the traditional use of specified preparations of *Juglans regia* leaves in a monograph of the European Community. European walnut leaves fullfils the requirement of therapeutic use for at least 30 years (15 years within the Community, Directive 2004/24/EC).

Indications for treatment: 1) Traditional herbal medicinal product for mild superficial inflammatory conditions of skin. 2) Traditional herbal medicinal product used in excessive perspiration of hands and feet.

Due to the lack of data on acute and chronic toxicity, repeated dose toxicity, mutagenicity, carcinogenicity, reproductive and developmental toxicity, a list entry for Juglans regia leaves cannot be recommended.

Benefit / Risk Assessment

There are some concerns about serious side effects with *Juglans regia* leaves due to presence of juglone. However, juglone is unstable and in dry leaves is present only in traces.

There are reported side effects concerning allergic reactions due to the *Juglans regia* leaves preparations used. No serious adverse events with a therapeutic posology of the herbal preparations are reported.

Despite the insufficiency of toxicological data base, levels of exposure associated with the use of walnut leaves by topical route of administration for limited time most probably has no relevance to human health and do not results in significant risk.

It can be concluded that the benefit/risk assessment for *Juglans regia* leaves preparations is positive for use in therapeutical dosages in specific conditions of the mild superficial inflammatory conditions of skin and in excessive perspiration of hands and feet.

Annex

List of references