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Dedicated to the memory of
Professor Ecaterina Ciorănescu-Nenitzescu (1909–2000)

COLLAGEN-THUJA TINCTURE BIOMATERIALS FOR WOUND TREATMENT

1. ANALYSIS OF THE VOLATILE FRACTION FROM THUJA COLUMNARIS TINCTURE BY GC-MS

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Thuja occidentalis var. columnaris tincture for topical use was prepared by extracting its active phytochemicals in 96% alimentary ethyl alcohol solution in view of obtaining collagen-thuja tincture dressings for wound treatment. The chemical composition of the volatile components was determined by GC-MS. A total of 39 peaks were obtained, of which 36 were identified. Terpene and terpenoids represent 88.4% of the volatile compounds of the tincture, from which 47.4% α-thujone, 8.3% β-thujone and 10.3% fenchone. The rest of 11.6% consists in pimaric acid – 5.6%, phytol – 4.4% and fatty acids (oleic, palmitic and linolenic) esters – 2.6% as main components as well as small amounts vitamin A and E acetates, plant hormone Gibberelin A 1 and cyclohexanone. All the volatile compounds are more or less antimicrobial and antifungal, excepting cyclohexanone, which might be a warranty for a high efficiency of thuja-collagen biomaterials as wound healing dressings.

INTRODUCTION

Collagen, due to its biological characteristics, such as full biodegradability, restorability, weak antigenecity and haemostatic properties, possesses excellent biocompatibility and safety, which makes it the primary source in biomedical application,[1,2] including wound healing.[3,4] In the form of sponge[5] or sheet[6] it is useful as a temporary cover for the treatment of different wounds: pressure sores, skin loss secondary to burns, trauma, amputation, chronic ulcer, leprosy, or skin graft sites.

The collagen capacity to heal wounds is due to its property to adhere to the wet wounds, absorb large amounts of tissue exudates, preserve a moist environment, and stimulate the formation of new granulation tissue and epithelium on the wound.[7,8] It also serves as a template for the infiltration of fibroblasts, macrophages, and lymphocytes and attracts additional monocytes to the wound.[6] Nevertheless, being a protein, it is a substrate for bacteria and can not promote by itself the healing of an infected wound. But associated with antibiotics and applied topically it functions as a drug delivery system, controlling the wound infection by localized delivery.[9-11]

Thuja occidentalis leaf and twig preparations, used by the Native Americans to prevent or treat scurvy, led to the tree's being called tree of life or arbor vitae.[12,13] More recent research has shown that hydroalcoholic extracts or tinctures in fact have antiviral, anti-inflammatory, and antibacterial properties against both Gram- negative and Gram-positive bacteria. They were applied externally to treat external fungal infections of the skin (ringworm and thrush), remove anal or genital warts and eczema,[12] remedy sores, wounds and rashes,[14] sloughing wounds, ulcers, bedsores, senile and other forms of gangrene.[15] Due to its

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pronounced antimicrobial properties, *Thuja occidentalis* tinctures can be used topically in dressings for healing of many kind of infection, particularly those characterized by a foul smelling, necrotic discharge.[16] Moreover, it seems to change the tissues, involving the epithelial and subepithelial structures.[17]

Considering the wound healing properties of collagen and Thuja hydroalcoholic extracts, the combination of collagen with Thuja tincture by its incorporation into collagen gels and preparation of sponges or sheets might result in performing dressings for the treatment of infected dermal wounds.

The secondary metabolites of *Thuja occidentalis*, like those of many other plants, depending on genus as well as on the environmental factors as light intensity and duration, temperature, elevation, season, soil and nutrition elements,[18,19] a large variety of ecotypes can be distinguished. That is why we prepared our own tincture using leafs and twigs of *Thuja occidentalis var. columnaris*, ecotype harvested from Cluj area (Rădaia), used by the homeopathic drugs manufacturer PlantExtrakt.

The objective of the present paper is the preparation of Thuja tincture and identification of the main antibacterial and antifungal volatile components, in view of estimating the wound healing capacity of collagen-*Thuja* tincture wound dressings from the healing action of its components. Moreover, synergistic effects might be observed.

RESULTS

Thuja occidentalis tincture has a content of dry substance of 3.705 %. Its gas chromatogram, shown in Figure 1, reveals the presence of 39 volatile components from which we were able to identify 36, having concentrations ranging between 0.1 and 47.7% if only the volatile compounds of the tincture that can be emphasized by gas chromatography are considered.

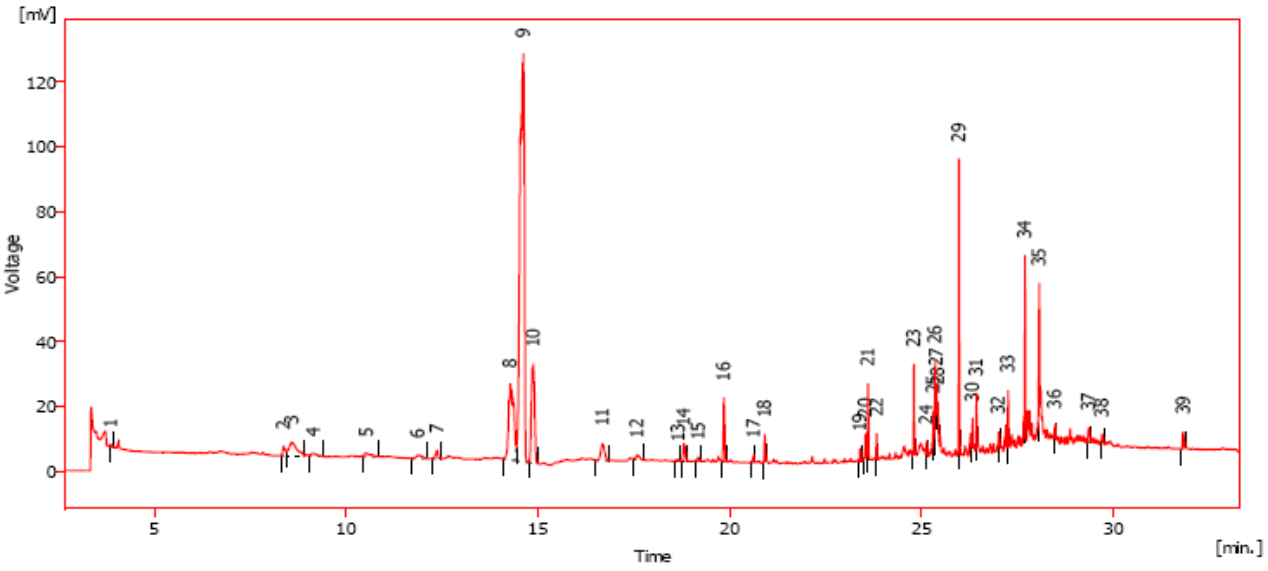

Fig. 1 – Gas chromatogram of *Thuja occidentalis var. columnaris* tincture.

The identified compounds, their retention times, percent concentrations and identity of spectra with those in the NIST Mass Spectral Search Program, version 2.0/2006 for the NIST/EPA/NIH mass spectral library are given in Table 1.

Table 1

Peak number, compounds identified, their retention times, relative area percentages and spectral fit

Peak no.	Compound	Retention time, min	Percentage	Spectral fit %
1	Calcitritol	3.723	1.1	86.2
2	Caryophilene	3.873	0.1	89.2
3	Sabinene	8.377	0.6	88.9
4	β-Terpinene	8.590	2.8	92.1
5	β-Myrcene	9.157	0.3	94.7

6	Limonene	10.535	0.7	91.2
7	γ-Terpinene	11.882	0.6	93.4
8	Fenchone	14.284	10.3	97.1
9	α-Thujone	14.625	47.4	93.4
10	β-Thujone	14.881	8.3	91.7
11	Camfor	16.689	1.7	87.2
12	Phenchyl acetate	17.589	0.5	88.4
13	α-Terpineol	18.660	0.1	88.9
14	Camphene	18.809	0.9	86.2
15	Bornyl acetate	19.850	2.1	88.7
16	Thymol	20.626	0.2	85.1
17	Terpineol acetate	20.912	0.6	86.4
18	Germacrene D-4-ol	23.394	0.3	87.2
19	Caryophillene epoxid	23.535	0.4	85.2
20	Trans-pinocarveol	23.603	1.4	87.6
21	Linolein	23.829	0.5	92.4
22	Cyclohexanone	24.801	1.5	94.1
23	Coniferol	25.130	0.2	87.3
24	(-)-Spathulenol	25.309	0.4	85.9
25	Palmitic acid ethylester	25.356	1.0	88.9
26	Beyerene	25.386	0.4	94.7
27	Phytol	25.974	4.4	95.2
29	Linolenic acid ethylester	26.328	0.4	91.4
30	Linolenic acid methylester	26.431	0.7	92.8
31	Vitamin A acetate	27.258	1.0	90.6
33	Totarol	27.697	2.6	93.7
34	Pimaric acid	28.068	5.6	95.3
35	Gibberelin A 1	28.478	0.4	91.5
36	Vitamin E acetate	31.822	0.6	90.4

DISCUSSION

Wound infection is a major problem, many types of wounds being prone to microbial contamination leading to infection. Treating infected wounds reduces the bacterial burden and removes thus a barrier to healing.

Topical application of *Thuja occidentalis* tincture incorporated into collagen sponges and sheets might be an efficient therapy to destroy microbial population due to the release of its active components that have antiviral, anti-inflammatory, and antibacterial properties and the booster healing by the collagen.

It is well established that terpenes and terpenoids – components of the essential oil of thuja occidentalis that give the fragrance of plant – are active against bacteria[20-24] and fungi.[24-26] That is why only the volatile fraction of the tincture was analysed.

According to Harnischfeger and Stolze[27], cited by Naser *et al*.,[28] the composition of the fresh plant (related to the dry substance) is: 0.6% essential oil, 2.07% reducing sugar, 4.9% water-soluble polysaccharides, 2.11% water-soluble minerals, 1.67% free acid and 1.31% tannic agents, but there are no details about the ecotype or season of harvesting (probably Germany).

The Table 1 shows that our tincture contains mainly terpenes and terpenoids – 88.4%, as well as other compounds: a resin acid (pimaric) – 5.6% [(1*R*,4a*R*,4b*S*,7*S*,10a*R*)-1,4a,7-trimethyl-7-vinyl-3, 4, 4b, 5, 6, 9, 10, 10a-octahydro-2*H*-phenanthrene-1-carboxylic acid], fatty acids (oleic, palmitic, linolenic) esters – 2.6%, cyclohexanone – 1.5%, calcitritol (a form of vitamin D3) – 1.1%, vitamin E acetate (tocopheryl acetate) – 0.6%, and coniferol (4-hydroxy-3-methoxycinnamyl alcohol) – 0.2%.

The main component of the tincture is α-thujone [4-methyl-1-(1-methylethyl-2-yl-bicyclo[3.1.0]hexan-3-one, 47.4%)], followed by fenchone (1,3,3-trimethyl-2-norcamphanone, 10.3%), β-thujone [4-methyl-1-propan-2-yl-bicyclo[3.1.0]hexan-3-one, 8.3%, α- to β-thujone ratio 85/15 – in agreement with the European Agency for Evaluation of Medicinal Products – EMEA], pimaric acid (7-ethenyl-1,4a,7-trimethyl-3,4,4b,5,6,9,10,10a-octahydro-2H-phenanthrene-1-carboxylic acid, 5.6%) and phytol (3,7,11,15-tetramethyl-2-hexadecen-1-ol, 4.4%), which constitute 76.0% from the total volatile compounds, while those having the concentration of 1% and higher represent 90.2% (see Table 1).

The presence of the fatty acid esters can be explained by their origin, common with that of terpenoids, the both classes being synthesized from acetate units.

The volatile components of our tincture, common with those obtained from some other ecotypes of *Thuja occidentalis,* are shown in Table 2.

As can be seen from Table 2, the composition of the volatile fraction of our tincture is very similar with that of the essential oil of *Thuja occidentalis* malonyana from Slovakia[19] and of the dried plant specified by Naser.[28]

The pharmacological properties of *Thuja occidentalis* are typically attributed to the essential oil, especially thujone, which has antimicrobial properties (EMEA 1999), so all the terpenes and terpenoids from Table 1 proved to be more or less antimicrobial.[25,29]

Table 2

Some *Thuja occidentalis* ecotypes and their main secondary volatile metabolites

Compound/*Thuja* ecotype	Naser *et al*.[28]*	Slovakia[19]**	http://www.toddcaldecott.com***	Nigeria[29] ***	http://www.chanchalcabrera.com***	Present paper
Borneol	+	-	+	-	+	-
Bornyl aceyate	-	+	-	-	-	+
Camphene	+	+	+	+	-	+
Camfor	-	+	+	-	+	+
Fenchone	+	+	+	-	+	+
Germacrene D	-	-	-	+	-	+
Limonene	+	+	-	+	+	+
Myrcene	+	+	+	+	+	+
Sabinene	-	+	-	+	-	+
α-Terpine	+	+	-	-	-	-
Terpineol acetate	-	+	-	-	-	+
Terpinolene	+	-	-	-	-	+
α-Thujone	+	+	+	-	+	+
β- Thujone	+	+	+	-	+	+
Thujylalcohol	+	-	+	-	-	+
Thymol	-	+	-	-	-	+

* Dried plant; **essential oil, *** essential oil, not all compound specified

Pimaric acid, the fourth from the point of view of concentration of volatile compounds in the tincture, has antibacterial effects especially for Gram-positive bacteria.[30] Methyl and ethyl fatty acid esters have also antibacterial and antifungal activity,[31] while vitamin A and E acetates are well known to be beneficent for the skin. Consequently, all the volatile components of the tincture, excepting cyclohexanone, support the wound healing process.

Considering the healing capacity of collagen, the antibacterial and antifungal properties of tincture volatile components, the high percentage of α- and β-thujone (55.7%), high efficiency is expected for the collagen dressings obtained combining collagen gels with Thuja tincture.

EXPERIMENTAL

Preparation of *Thuja occidentalis* tincture. Fresh leaves and twigs of *Thuja occidentalis* L – columnaris harvested at the end of April 2009 from Rădaia – Cluj, Romania, were ground immediately to a coarse powder and the tincture for topical use was prepared by maceration in 96% ethanol aqueous solution at a strength (*Thuja*/menstruum ratio) of 1/2[32] and a temperature not exceeding 25°C for 10 days in the absence of light. [33] Its concentration, obtained by evaporation at 105°C, was 3.705% (m/v).

Analysis of tincture. The tincture was analysed using a GC-MS system consisting of a HRGC 4000B gas chromatograph in combination with a KMASS-5 autosampler and a Konik MSQ 12 quadrupole mass selective detector provided with prefilters. Data acquisition and analysis were performed using MS DataRed software supplied by the manufacturer, Konik-Tech, Spain. The clean-up step was applied by passing 1 mL of tincture through a SPE C18 column washed previously with 1 mL ethyl alcohol and 1 mL deionized water. The column was eluted droplet by droplet with 1 mL ethyl alcohol. Continuing the elution with another 1 mL, no components appear in the chromatogram. The operation was done in 10 columns. The 10 resulted ethanol solution were injected separately into a Macherey Nagel Optima 35MS, silarylene stationary phase with medium polarity, 30 m x 0.325 mm i.d., film thickness 0.25 μm capillary column, using the following temperature program: 50°C hold for 5 min, 5°C/min up to 120°C, hold for 5 min, 20°C/min up to 300°C, hold for 5 min. Split/splitless injection system with a split ratio of 1/20 with Electronic Pressure Control (EPC) was used in order to get the requested flow accuracy and retention time reproducibility.

The temperature for the injection port, transfer line and ion source was set at 230, 310 and 250°C, respectively. Split/splitless injection mode with a split time of 1 min and

helium with a constant flow rate of 1.0 mL/min as carrier gas was used, injecting 1 μL of sample.

To determine the retention times and characteristic mass fragments the total ion current, TIC, chromatogram was recorded and the electron impact mass spectra of the compounds were collected at 70 eV. Quantitative data were calculated from peaks total area percent of the TIC chromatogram using the apparatus software (Clarity Chrome software package). Similar results were obtained for the 10 chromatograms. The two diastereomers α- and β-thujone were baseline separated (retention times 14.6 and 14.9 min, respectively), as can be seen from Figure 1 and Table 1. To verify the ratio α- to β-thujone given by chromatogram, the chosen diagnostic mass fragments (m/z 81, 110 and 152) were monitored in the selected ion detection - SIM mode. For comparison α- + β-thujone certified reference material (Sigma-Aldrich) was also analysed.

CONCLUSIONS

Thuja occidentalis var. columnaris tincture was obtained by the maceration of ground leafs and twigs in 96% alimentary ethyl alcohol for 10 days at temperature no exceeding 25°C.

GC-MS analysis of the volatile components of the tincture shows that 88,4% are terpenes and terpenoides, from which 55.7% are α- (47,4%) and β-thujone, followed by fenchone (10.3%), while 5.6% consists of pimaric acid, 4.4% of phytol and 2.6% of fatty acid esters as main components, all of them having more or less antibacterial activity. Other components are vitamin A (1.0%) and E (0.6%) acetates, plant hormone Gibberelin A 1 (0.4%) and cyclohexanone (1.5%).

All the volatile compounds having more or less antimicrobial properties, excepting cyclohexanone, high efficiency is expected for collagen-based biomaterials containing thuja tincture as wound healing dressings.

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