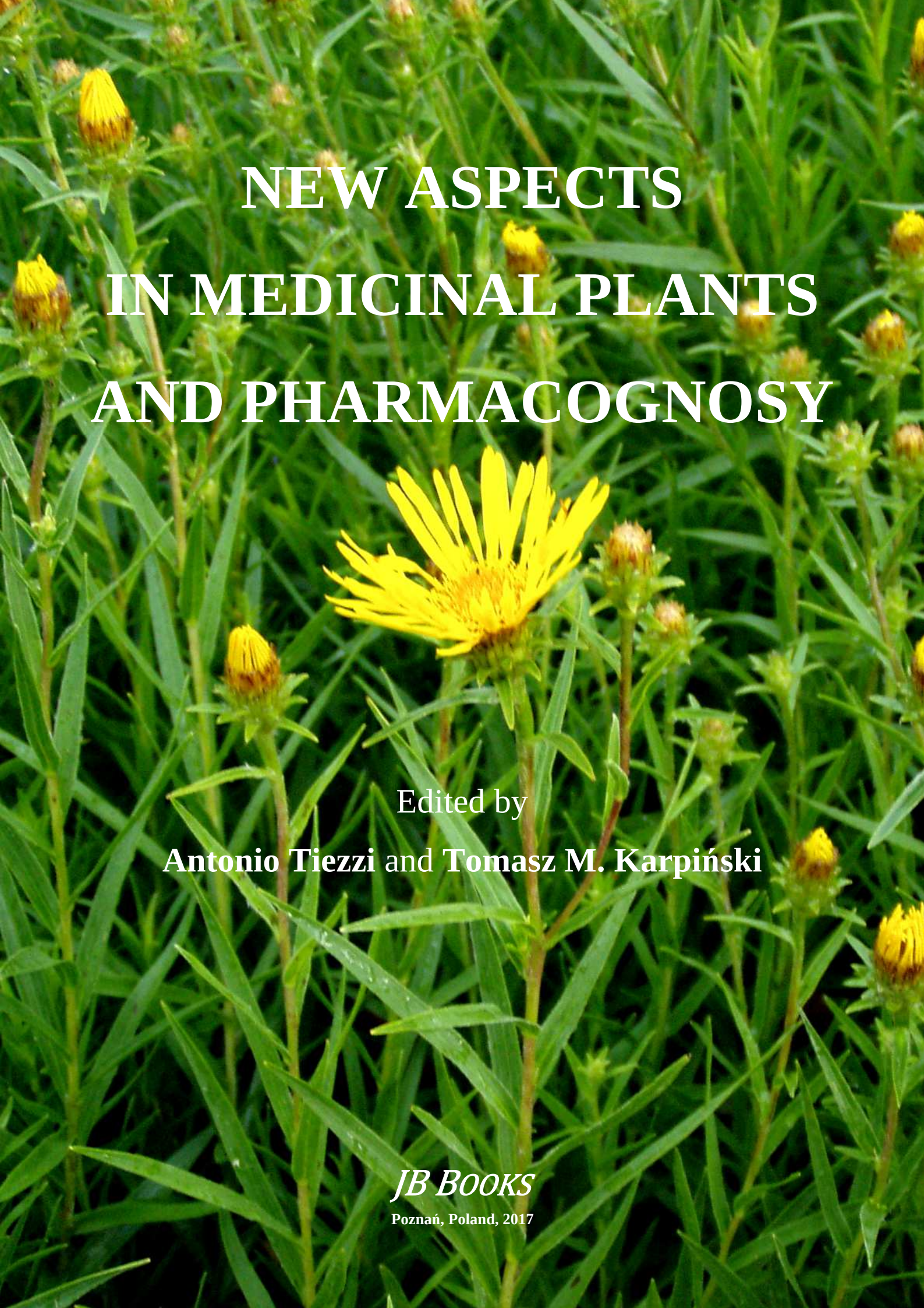

NEW ASPECTS IN MEDICINAL PLANTS AND PHARMACOGNOSY

Edited by

Antonio Tiezzi and **Tomasz M. Karpiński**

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Pharmacognostic and chemical studies of *Pimenta racemosa* (Mill) J.W. Moore that grows in Ecuador

1

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ABSTRACT

P. racemosa (Mill) J.W. Moore, belongs to the genus *Pimenta* of the Mirtaceae family, is native to the Lesser Antilles, Puerto Rico and Cuba, being widely cultivated in the American Tropics. The essential oil extracted from the leaves has disinfectant and astringent properties and does not give rise to allergic reactions on people. In this work the pharmacognostic and chemical study of the species that grew in the city of Guayaquil, Guayas province (Ecuador), was carried out, on which it was possible to establish the macro and micromorphological characteristics of the species, that were researched for the first time, The physical-chemical quality parameters of the leaves of the species were also determined, aspects that were previously reported. A comparative study was carried out on the extraction of essential oil from the leaves using the hydrodistillation method and the microwave assisted hydrodistillation (MWHD) method, for which a yield of 1,3% was obtained, which was higher than that of which was reported in the bibliography. The distillation time to reach this

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yield was lower when MWHD was used and some differences were observed in the physicochemical characteristics of the essential oil. The chemical composition of the essential oil determined by the use of the GC-MS coupled system made it possible to identify methyl eugenol and estragol as the major components, followed by β-pinene, 1,8-cineole, linalool and limonene, which correspond to the information reported for *P. racemosa* var. racemosa, although modifications in the qualitative-quantitative composition were evident, depending on the extraction method.

Keywords: *Pimenta racemosa*; Pharmacognostic study; Hydrodistillation extraction; Microwave assisted hydrodestillation.

1. INTRODUCTION

Ecuador is an area rich in natural resources and abundant production of aromatic plants as well as being considered as one of the most biodiverse countries in the world. Thanks to its privileged geographic location in the neo-tropic it is of great influence in its environment to create most diverse life forms of flora and fauna [1].

There is a great variety of aromatic plants in the country with very little research published and this has been the guideline to the beginning of this project.

The species *Pimenta racemosa* var. racemosa (Mill.) J.W. Moore, belongs to the genus *Pimenta* of the Myrtacea family, is native to the Lesser Antilles, Puerto Rico and Cuba, being widely cultivated in the American Tropics. It is commonly known as Bay-Rum Tree, Malagueta, Nugget and Pepper and has been studied due to the contents in its leaves of volatile oils, which once distilled, are used in the manufacture of cosmetics, mainly in elaboration of aftershave, soaps, colonies and treatments for hair [2, 3].

This essential oil also has anti-inflammatory and analgesic properties [4-6] and is also used as febrifuge [7] and for its nematicidal activities [8] and insecticide [9], the most outstanding biological properties being the antimicrobial effects explained by the high percentage of phenols [10-12].

Essential oils are secondary metabolites of plants, which are contained in secretory glands or vesicles immersed in the tissue of different plant organs, such as leaves, flowers, barks, fruits and roots [13].

To obtain the essential oils, there are different methods, distillation being the most common method, since it is the most economical and can be carried out by steam trapping or hydro-distillation. Both techniques have proven to have the quality of being friendly to the environment, thanks to the minimum impact generated by using water as a solvent, which is beneficial for the industrialization of the method and for economic growth [14].

The constant evolution of analytical chemistry has allowed the development of techniques such as hydro-distillation assisted by microwave radiation, which causes localized heating by contacting the plant material in water with the reactor. Therefore, its operating time is lower compared to the traditional method and, in addition, an increase in the yield of the essential oil and a decrease in the costs of production can occur [15].

The main objective of this chapter is to compile the results of the pharmacognostic and phytochemical studies carried out on the leaves of *Pimenta racemosa* (Mill) J.W. Moore, who grows Ecuador.

2. MATERIALS AND METHODS

2.1. Gathering of plant material

The fresh leaves of the species *P. racemosa* were collected in February and October 2016, during morning time at the Botanical Garden north of the city of Guayaquil, Ecuador. The botanical identification was made by Dr. Carmen Bonifaz of Elao, of the Guay Herbarium, Faculty of Sciences, assigning the identification number 008 and the code GUAY 13,100.

2.2. Macroscopic evaluation

For the study of the macro-morphological traits, samples of 50 leaves were analyzed and through the use of a digital vernier calibrator, the length and width of the samples were determined. These were observed with a magnifying glass to describe the shape of the limbus, the base, the apex, the border and the nerves. Also the characteristics of the leaves by the beam and the underside and the presence of the petiole were taken into account. To do so, the aspects raised by Miranda and Cuéllar [16] were taken into account.

2.3. Morphological and anatomical evaluation

For the micro-morphological study, the inclusion in parafine was carried out, for which the methodology described by Miranda and Cuellar [16] was used.

Small cuts of the leaves were made, by the middle nerve of the leaves. The small cuts were subjected to dehydration with 45%, 60%, 75%, 98% ethanol for 48 hours each at room temperature. Finally they were deposited for 48 hours in the intermediate reagent (hexane). After the dehydration was carried out, the samples were deposited in paraffin in a Memmert oven at 60°C for 2 hours, then the Leuchart sheets were prepared, the paraffin was added in the molds and the sample was placed, waiting for its solidification.

Transverse cuts were performed for the histological analysis. The cuts were performed on a microtome, dewaxed and then hydrated and rinsed with sodium hypochlorite. They were stained with 1% safranin and fixed with glycerinated gelatin [17-18].

A Nikon Alphaphot-2 microscope with TK-C1380 JVC color video camera model TK-C1380U was used to visualize the different internal anatomical traits of the plant.

2.4. Physicochemical parameters of the leaves

The trials were performed following the methodology described by WHO [19]. These were:

2.4.1. Residual moisture

The azeotrope method was used, measurements were made in triplicate from 10 g of dry leaves, a Dean-Stark kit coupled to a 1 L balloon was used. Toluene was used as the solvent.

2.4.2. Total ashes

For the analysis, a MLW Eliktro muffle was used for 2 hours, the weights were performed on a Kern model analytical balance model: ABS-220-4. Each determination was done in triplicate from 2 g of sample.

2.4.3. Soluble substances

This trial was determined in triplicate, starting from 5 g of the drug. The solvents used were water and 98% alcohol.

2.5. Identification of the metabolites by chemical screening

Phytochemical screening was performed on dry leaves, according to the procedure described by Miranda and Cuellar [16]. A solvent extraction system of increasing polarity was used from the same plant material. The dried drug was successively extracted with diethyl ether, ethanol and water for 48 hours to obtain ethereal, alcoholic and aqueous extracts, which were subjected to different tests.

2.6. Obtaining and analysis of essential oil

The extraction of the oil was carried out by distillation, techniques of hydro-distillation (HD) and microwave assisted hydro-distillation (MWHD) were used. A 1000 ml capacity balloon was used and 500 g finely chopped fresh vegetable material was placed for each extraction.

Volume readings of essential oil obtained by both techniques were performed from 10 minutes and up to a time of 4 hours (240 minutes) to form the yield versus time curves. The process was repeated several times. For the MWHD was worked similar to the HD, but placing the balloon in a microwave oven Panasonic with a power of 1000 W, for a time of 240 minutes with cycles of 10 minutes. To the balloon, 250 ml of water and the fresh plant were added. The oils obtained for each were collected, dried with anhydrous sodium sulfate and stored in amber refrigerated vial for their chemical and chemical physical analyzes.

2.7. Physical-chemical analysis of essential oils

To the essential oils obtained by the different extraction methods, the following analyzes were performed, as described in the literature [16]:

- Refractive index
- Relative density

- Solubility in alcohol
- Evaporation residue.

2.8. Statistical analysis

The data was processed statistically using a multiple variance analysis (ANOVA) considering a significance level of 5% and with the help of the statistical software IBM SPSS version 22.

2.9. Gas chromatography/mass spectrometry analysis

The essentials oils were analyzed by using a gas chromatography mass spectrometry equipment Agilent Technologies (7890A GC system and 5975C inert XL MSD with triple axis detector). A capillary column DB-5ms UI (30 m x 0.25 mm x 0.25 µm) and helium as the carrier gas (1.1 ml/min). The injection of 1.0 µl of sample diluted in hexane was done at a temperature of 250°C with split mode, split ratio 25:1, the detector temperature was 230°C and the oven temperature was maintained at 60°C for 1.0 minutes, then it was increased to 240°C at 2°C/min. The electron ionization to 70 eV and 230°C was used as ion source and the data compounds were collect with the full scan mode (40-1000 µma). Finally, compounds were identified by comparison of their mass spectra and mass reference of Wiley 9th with NIST 2011 MS Library.

3. RESULTS AND DISCUSSION

3.1. Botanical characterization of the species

The botanical characterization of the plant material is the first aspect to be analyzed in a pharmacognostic study, with the objective of establishing the macro and micro-morphological characteristics of the species under study.

3.1.1. Macro-morphological evaluation of leaves

The foliar sheet of coriaceous consistency, presented a bright dark green color by the

beam, with smooth surface; The pubescent underside of light green color, elliptical form, palminervia, whole edges, petiolate without presence of trichomes, obtuse base and obtuse apex; Points of oil glands are observed on the underside (Figure 1). The characteristics are in correspondence with the reported [20].

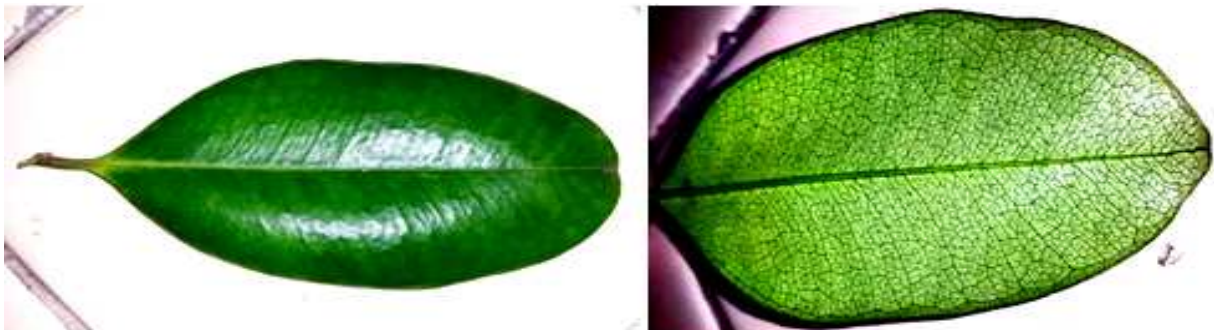

Figure 1. Macrophormological characteristics of the leaves

In regards to leaf dimensions in the study population, these showed a variation within a range of 4.8 to 13.7 cm for the length and 3.4 to 6.8 cm for the width. The results obtained are consistent with those reported in the literature [20-21], which indicate a range between 4-10 cm for the length and 2.5-6 cm for the width, and the values obtained in this study (8.64 ± 1.9 cm, long and 4.69 ± 0.88 cm, width), within this range of values.

3.1.2. Micro-morphological evaluation

In the leaf anatomy at the level of a transverse section of the central nerve (Figure 2) it was observed that the abaxial surface is completely concave and the adaxial is slightly convex. In the innermost part of the central nerve, it was observed that it is occupied by a large number of conducting beams (HC) resembling the forms of spinal radii in the dorsiventral direction, surrounded by a pericycle of parenchymal cells (Pa). Closer to the lower surface of the nerve is the colenchyma (Co) and the abaxial epidermis (EAb).

In the structure of the lateral arms (BL), the epidermal tissue was observed in its adaxial part (EAd) formed by a stratum of cells. And then below this epidermis is the palisade parenchyma tissue (PE).

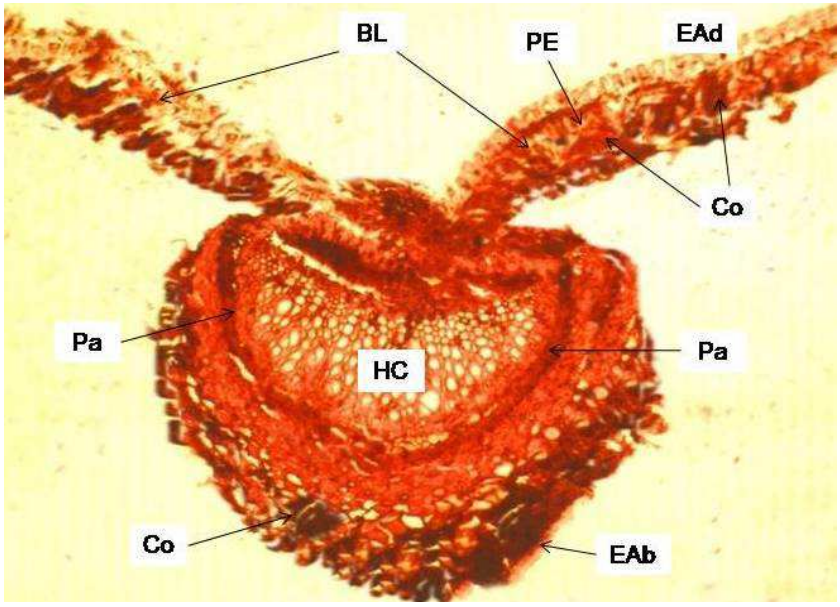

Figure 2. Micro-morphological details of the central nerve of the leaf. BL: lateral arms; PE: parenchyma in palisade; EAd: adaxial epidermis; Co: colenchyma; HC: conductive beams; EAb; epidermis abaxial

Figure 3 shows several enlarged sections of one of the side arms. In Figure 3 the adaxial epidermis (EAd) was formed by a stratum of tabular cells, then a tissue in palisade (PE) columnar cells. In Figure 3 B the colenchyma (Co) formed a conglomerate of nearly rounded cells. Glands, essential oil containers and some calcium oxalate (Glan) crystals are also seen (Figure 3C). The results obtained for the micro-morphological characteristics are not described in the consulted literature.

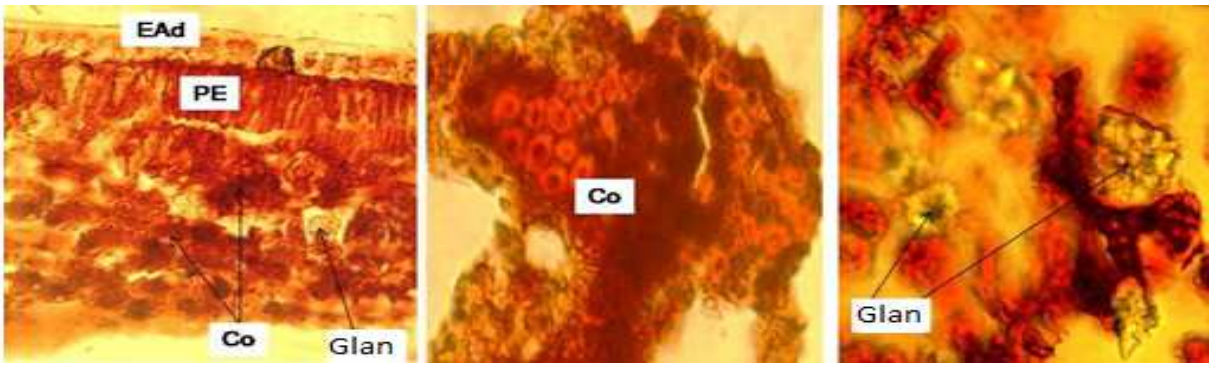

Figure 3. Extended sections of the micromorphological details of *P. racemosa* leaves. A: portion of a lateral arm; B: cells of the colénquima tissue; C: calcium oxalate crystals EAd: epidermis adaxial; PE: parenchyma in palisade; Co: colenchyma; Glan: essential oil glands

3.2. Determination of physicochemical parameters

Another aspect addressed in the study of the species was the establishment of the physicochemical parameters of the dry leaves (vegetal drug). The results are presented in Table 1.

Table 1. Physical chemical parameters determined to *P. racemosa* leaves

Parameter	Valor medio X	DS	CV
Residual humidity (%)	13,97	0,00	0,00
Total ash (%)	8,33	0,21	2,50
Substances soluble in alcohol (%)	19,59	0,27	1,38
Substances soluble in water	18,21	0,69	3,78

Legend: X = average value; DS = standard deviation; CV = coefficient of variation

The water content in the plant is one of the critical aspects and proves to be a key parameter for the conservation of the drug. For this parameter the Norms and Pharmacopoeias establish, depending on the plant organ, content between 8 and 14% [19, 22].

In the analysis performed the result obtained for the moisture content was 13.97% which is within the reference range, although in the somewhat high value could have influenced the fact of containing the leaves essential oil and have used the method of Dried at room temperature.

As for the total ash, they indicate the quality of the material with which it is worked and is an important basis for judging the purity and identity of the material. On the other hand they provide information on the possible adulteration of the drug with inorganic materials or foreign bodies which also depends on the mineral composition of soils [16]. The standards and pharmacopoeias establish a total ash index that is between 2-12% [19, 23]. The analysis of total ash to the plant material resulted in an 8.34% percentage that is within the established range.

Finally, the analysis of soluble substances in water and ethanol was performed at 98% and the results indicate that the percentages of soluble substances did not differ much that is to say that the plant contains both soluble substances in water and in ethanol.

3.3. Phytochemical screening

The results of the phytochemical screening carried out on ethereal, alcoholic and aqueous extracts of the leaves of the plant show a high variability of the present compounds, such as oils, fats, triterpenes steroids, lactones, coumarins, flavonoids, phenols, tannins, catechins, quinones, anthocyanidin, saponins and reducing compounds.

When analyzing the results obtained in the phytochemical screening carried out on each extract, they were found to be in agreement with those reported in the literature [24], where the abundant presence of phenolic compounds (tannins, flavonoids and quinones), as well as saponins and the absence of alkaloids.

3.4. Collection and analysis of essential oil

3.4.1. Essential oil yield

In the study of the dynamics of accumulation of essential oil as a function of time, it was observed that in order to obtain a yield of 1.4% by the HD method, a time of approximately 2 hours had to be employed, whereas by the MWHD method, a yield of 1.8% was obtained in only 60 minutes. Figure 4 shows the graph corresponding to the distillation dynamics of the essential oil by both distillation techniques.

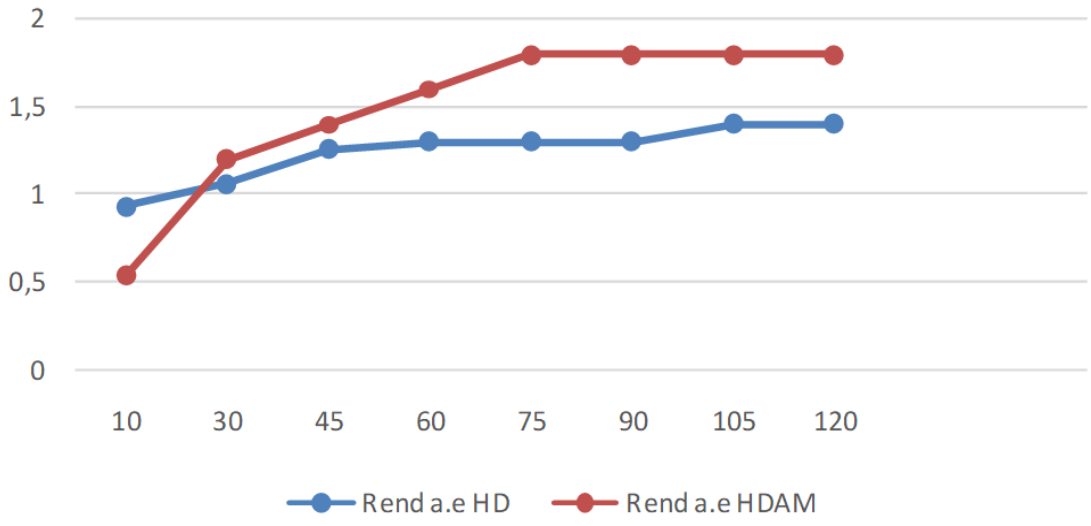

Figure 4. Dynamics of distillation of the essential oil by HD and MWHD methods

The oil content in fresh leaves ranges from 1-3% [25], so the results obtained are within the range reported. It should be noted that in a study by Bustamante et al. [26] from the species collected in the same area, but in February, the yield reached by HD was 1.3%, which shows that the harvesting time influences the yield of essential oil.

3.4.2. Physical-chemical constants of essential oil

The essential oils obtained by both techniques were determined some physical-chemical parameters and the results are presented in Table 2.

Table 2. Physicochemical parameters of the essential oil obtained by both distillation techniques

Physicochemical characteristics of essential oils					
Technique	Refraction Index	Density	Solubility		Evaporation Residue
			Alcohol 70%	Alcohol 80%	
Hydro-distillation	1.5075	0.9601	insoluble on 10 ml	soluble on 5 ml	0.1509
Hydro-distillation assisted by microwave	1.5139	0.9641	insoluble on 10 ml	soluble on 2.1 ml	0.0113

As observed, some differences were found between the essential oils obtained by the different techniques tested. The refractive index and the density were slightly higher for the oil obtained by MWHD, while it showed higher solubility in alcohol at 80% and lower heat of evaporation residue than that obtained by HD.

These differences in physicochemical parameters are an indication of possible differences in the chemical composition of these oils.

3.4.3. Chemical composition of essential oils

The analytical gaseous chromatogram of the essential oils obtained by the different extraction techniques is presented in Figure 5.

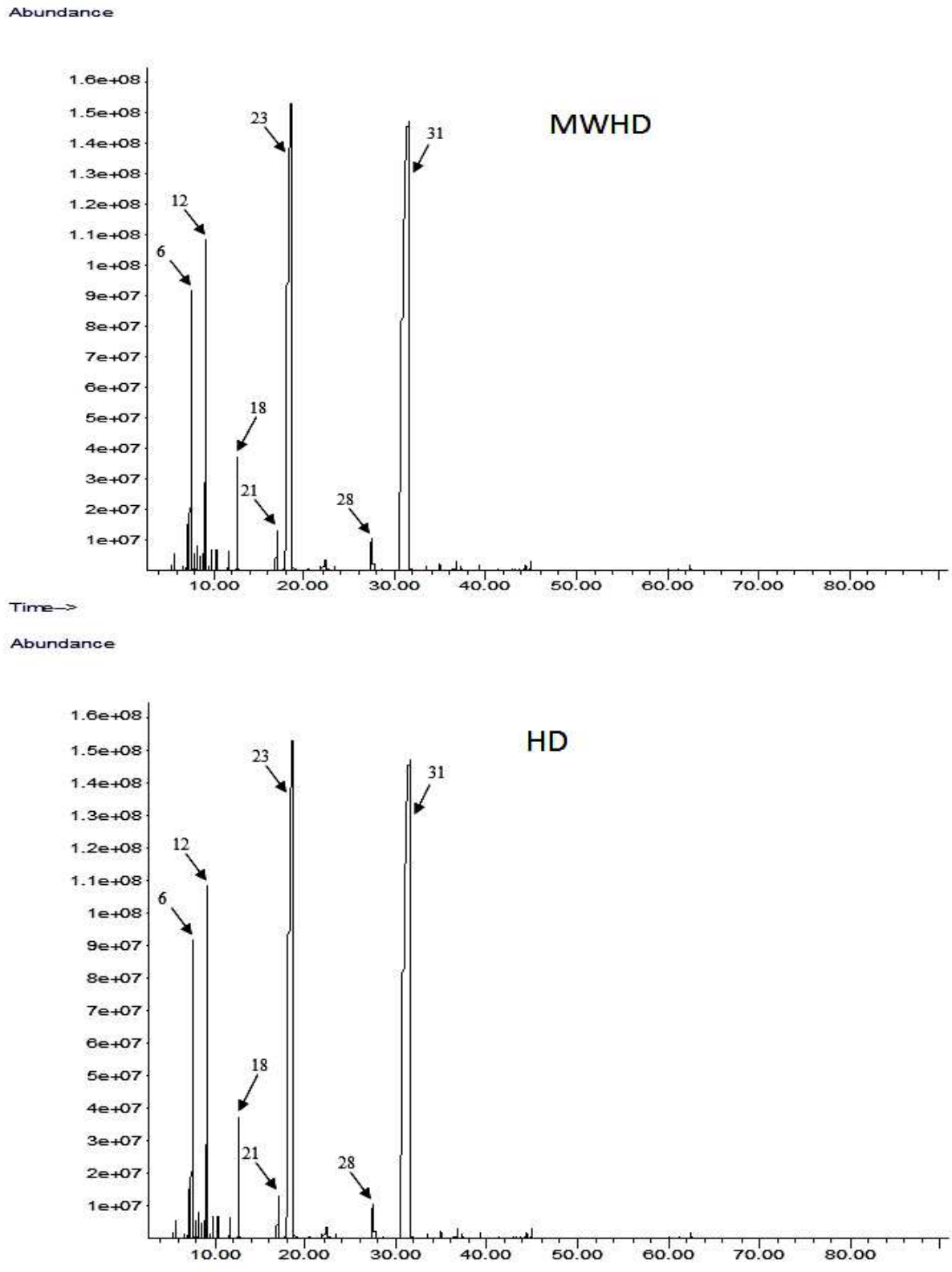

Figure 5. Analytical gaseous chromatograms of essential oils obtained by HD and MWHD

As observed, the main differences observed are in the intensities of the chromatographic peaks, which are somewhat superior in the microwave assisted distillation.

The chemical composition for these oils was determined by comparing their mass spectra with those of the equipment library and their comparison with the retention indices. It can be observed that of the 47 compounds to which structures were assigned, 24 correspond to monoterpene hydrocarbons and 23 to oxygenated compounds including terpenes and aromatics.

The major components of the essential oil were methyl eugenol with 47.50% relative abundance for HD and 50.82 for MWHD and estragol with 32.69% and 31.62% for HD and MWHD respectively, Both aromatic compounds; The β-pinene, monoterpene hydrocarbon, presented 7.01% and 5.81% of abundance for HD and MWHD respectively and 1,8-cineol, linalool and limonene, were the other three compounds with relative abundance higher than one percent (Table 3).

For the species *P. racemosa*, five varieties have been reported: racemosa, grisea, hispanoliensis, ozua and terebenthina, which are endemic to some areas of the Caribbean [28]. Of these, the racemosa variety has been the most cultivated and its essential oil marketed. However, according to geographic origin, the composition of this essential oil may vary fundamentally from the majority compounds, with the following compounds being mentioned for some commercial oils: eugenol + isoeugenol (33,8-56,2%), myrene (13, 9-31.6) and chavicol (8.9-21.6%) [29-32], other reports of commercial oils indicate methyl-eugenol, β-caryophyllene and α-humulene as major [7, 33].

For non-commercial essential oils, these authors have stated that the compositions have been varied, being able to determine as a majority the estragol and methyl eugenol; (53.1%), and mellenum (31.6%) and myrene (12.0%), and in others, geraniol (53.2%) and nerol (32.6%).

Leyva [34] points out, however, another composition for the essential oil obtained in Pinar del Río, Cuba, where the majority of the oil is not the aromatic compounds but the terpenoids, reporting a composition where terpinen-4-ol (20.7%), 1,8-cineole (20.4%), eugenol (10.7%), chavicol (10.1%) and α-terpineol (10.0%).

For the essential oil obtained from *P. racemosa* growing in Merida, Venezuela, Contreras [35] found that the main components were eugenol (48.7%), 1,8-cineole (12.7%) and limonene (13, 7%).

These observations show the great variability of the essential oil of *P. racemosa* in its chemical composition, depending on the climatic zone where it was studied. In this work, methyl-eugenol and estragol were the major components, followed by β-pinene, 1,8-cineol, linalool and limonene. These results are similar to those reported by Tucker [28].

Table 3. Chemical composition of essential oils obtained by HD and MWHD

No	Components	RIlit[a]	RI[b]	% HD	% MO
1	Thujene	932	921,80	0,03	0,03
2	α-Pinene	936	928,87	0,11	0,10
3	Camphene	950	944,53	0,01	0,00
4	1-Octen-3-ol	962	978,54	0,62	0,62
5	β-Pinene	978	990,75	7,01	5,81
6	Octanal	981	1002,45	0,10	0,12
7	l-Phellandrene	1002	1005,01	0,16	0,16
8	α-Terpinene	1013	1014,04	0,10	0,09
9	o-Cymene	976	1021,32	0,21	0,16
10	D-Limonene	1025	1026,33	1,38	1,10
11	1,8-Cineol	1024	1029,14	4,02	3,85
12	(E)-b-Ocimene	1041	1041,96	0,18	0,16
13	γ-Terpinene	1051	1053,12	0,17	0,17
14	trans-Linalooloxide	1058	1065,84	NI	NI
15	Terpinolene	1082	1080,94	0,18	0,16
16	Perillen	1090	1095,95	0,01	0,00
17	Linalool	1086	1100,85	1,44	1,40
18	Nonanal	1076	1103,52	0,01	0,01
19	α Terpineol	1176	1166,70	0,01	0,01
20	4-Terpineol	1161*	1176,42	0,59	0,57
21	Estragole	1175	1204,37	32,69	31,62
22	Decaldehyde	1180	1206,80	0,18	0,16
23	Z-Citral	1215	1234,65	0,01	0,01
24	Chavicol	1219	1264,48	0,27	0,52
25	Anethole	1262	1281,57	0,03	0,03
26	Eugenol	1331	1347,34	0,50	0,90
27	Ylangene	1376	1366,29	0,04	0,02
28	Methyleugenol	1369	1412,65	47,50	50,82
29	β-Ylangene	1420	1420,79	0,02	NI
30	Humulene	1455	1445,27	0,06	0,02
31	γ-Muurolene	1474	1466,35	0,02	0,00
32	Germacreme-D	1479	1470,66	0,14	0,07
33	γ-Elemene	1429	1484,33	0,01	0,00

No	Components	Rllit[a]	RI[b]	% HD	% MO
34	α-Muurolene	1496	1489,81	0,02	0,01
35	4-Allyl-2,6-dimethoxyphenol	1561	1495,47	0,03	0,02
36	α-Farnesene	1498	1500,85	0,17	0,12
37	δ-Cadinene	1520	1509,27	0,07	0,04
38	Elemicine	1522	1543,97	0,05	0,06
39	Spathulenol	1572	1564,69	0,01	0,01
40	Caryophylene oxide	1578	1567,25	0,02	0,01
41	Globulol	1589	1572,85	0,01	0,01
42	Junenol	1617	1606,50	0,03	0,01
43	Cubenol	1630	1615,51	0,03	0,02
44	tau.-Muurolol	1633	1633,27	0,10	0,03
45	α-Cadinol	1643	1644,32	0,15	0,13
46	Cembrene	1938	1937,68	0,06	0,02
47	4-(4'-Methyl-3'-pentenyl)-3-cyclohexenyl pentyl ketone	1967**	1986,53	0,11	0,08
	Total			98,69	99,23

Notes: [a]Rllit: retention indices according to tepenoids library Website [26] otherwise stated; [b]RI= retention indices measured on apolar capillary columns; NI= Non identified; *Retention index according to Bendahou et al. [27]; **Estimated in a non-polar column

Even if for HD and MWHD, the components of the essential oil do not vary in quality, they differ quantitatively. The oil obtained by MWHD presented a higher concentration of the major component (methyl eugenol), though for the rest of the components of greater concentration the percentage of abundance is slightly lower. Nevertheless, if we compare the yield obtained and the distillation time, it is evident that the technique that applies the MWHD, would be the best option for obtaining the oil of *P. racemosa*.

4. CONCLUSIONS

Through the pharmacognostic analysis made to the leaves of *Pimenta racemosa* (Mill) J.W. Moore, collected in the city Guayaquil, province of Guayas (Ecuador) the macro morphological characteristics of the species were established, which coincide with what has been reported in the literature.

The micro-morphological characteristics and quality parameters of the plant material were established for the first time.

The essential oils obtained by the techniques of hydrodistillation and hydrodistillation assisted by microwave presented differences in the yield and the time of extraction, being the technique that applies the radiations of superior microwave since it allows to obtain a better yield in less time.

The analysis of the chemical composition of the essential oil using the CG-EM coupled system allowed the identification of methyl eugenol and estragol as the major components of the essential oil, independent of the method of production used.

REFERENCES

1. Bravo E. Ecuador megadiverso: Apuntes Introductorios. Universidad Politécnica Salesiana. Editorial Universitaria Abya-Yala. Impreso en Quito-Ecuador. 2013.
2. Weiss EA. Spice Crops. CABI Publishing, USA. 2002.
3. Boning CR. Florida's best herbs and spices: native and exotic plants grown for scent, First edition. Pineapple Press Inc., USA. 2010.
4. Robineau L. Towards a Carabbean Pharmacopeia (Tramil 4), Enda Caribe, Santo Domingo, 1991: 287-295.
5. Fernández MA, Tomos MP, Garcia MD, De las Heras B, Villar AM, Saenz MT. Anti-inflammatory activity of abietc acid, a diterpen isolated from *Pimenta racemosa* var. racemosa. J Pharm. 2001; 53(6): 867-872.
6. Fernández A, Alvarez A, Garcia MD, Saenz MT. Anti-inflammatory effect of *Pimenta racemosa* var. ozua and isolation of the triterpene lupeol. Farmaco. 2001; 56(4): 335-338.
7. Ayedoun AM, Adeoti BS, Setondji J, Menut C, Lamaty G, Bessiere JM. Aromatic plants from tropica lWest Africa. IV. Chemical composition of leaf oil of *Pimenta racemosa* (Miller) J.W. Moore var. racemosa from Benin. J Essent Oil Res. 1996; 8: 207-209.
8. Park Il-K, Park Ju-Y, Kim KH Choi KS, Choi IH, Kim CS, Shin SC. Nematicidal activity of plant essential oils and components from garlic (*Allium sativum*) and cinnamon (*Cinnamomum verm*) oils against the pine wood nematode (*Bursaphelenchus xylophilus*). Nematology. 2005; 7(5): 767-774.
9. Noudogbessi J-P, Kossou D, Sohounhloué DCK. Effet Insecticide, Ovicide et Larvicide des Huiles Essentielles de *Pimenta racemosa* (Miller) et de *Chromolaena odorata* (L. Robinson) sur le Grand Capucin (*Prostephanus truncatus* (Horn)) du Maïs. J Soc Ouest-Afric Chimie. 2008; 26: 41-51.

10. Aurore GS, Abaul J, Bourgeois P, Luc J. Antimicrobial and antifungal activities of the essential oils of *Pimenta racemosa* var. racemosa P. Miller (J. W. Moore) (Myrtaceae). J Essent Oil Res. 1998; 10(2): 161-164.
11. Burt SA, Reinders RD. Antibacterial activity of selected plant essential oils against *Escherichia coli* O157: H7. Lett Appl Microbiol. 2003; 36(3): 162-167.
12. Saenz MT, Tornos MP, Alvarez A, Fernandez MA, Garcia MD. Antimicrobial activity of essential oils of *Pimenta racemosa* var. terebinthina and *Pimenta racemosa* var. grisea. Fitoterapia. 2004; 75(6): 599-602.
13. Miranda M., Cuéllar A. Farmacognosia y Química de los Productos Naturales. Editorial Félix Varela 2da Edición. 2012.
14. Ortega AF. Extracción, caracterización por CG-EM y actividad antibacteriana del aceite esencial obtenido mediante hidrodestilación de Passiflora edulis Flavicarpa de origen ecuatoriano. Tesis de Grado previo a la obtención del título de Ingeniero de Alimentos. Facultad de Ingeniería en Mecánica y Ciencias de la Producción. Escuela Superior Politécnica del Litoral. 2015.
15. Torrenegra ME; Granados C; Osorio MR y León G. Comparación de la hidro-destilación asistida por radiación de microondas (MWHD) con hidro-destilación convencional (HD) en la extracción de aceite esencial de *Minthostachys mollis*. Inf Tecnol. 2015; 26(1): 117-122.
16. Miranda M., Cuéllar A. Folleto de prácticas de laboratorio de farmacognosia y productos naturales. Editorial. Universidad de La Habana Cuba. Epígrafe I.3, V. 2000.
17. Gattuso MA, Gattuso SJ. Manual de procedimientos para el análisis de drogas en polvo. Editorial de la Universidad nacional de Rosario Urquiza. Argentina. 1999.
18. Khandelwal KR. Practical pharmacognosy. Techniques and experimentes. Twelveth edn. 2004.
19. QMS. Quality Control Methods foer herbal materials. Updated edition of Quality control methods for medicinal plant materials, 1998.
20. Royal Botanic Gardens, *Pimenta racemosa*. Kew: World Checklist of Selected Plant Families. Consultado el 15 de enero de 2016.
21. Blanco Fernández de CP, Espejo Serna MA, López Ferrari AR. Catálogo del Herbario de la Real Expedición Botánica de Nueva España (1787-1803) Conservado en el Real Jardín Botánico de Madrid. Catálogo general de publicaciones oficiales. Gobierno de España. CSIC. 2010.
22. Claus E. Farmacognosia. Ed. Editorial Revolucionaria. La Habana. Cuba 1-38. 1985.

23. Lou Zhi-Cen. General control methods for vegetable drugs. Comparative study of methods included in thirteen pharmacopoeias and proposals on their international unification. WHO/PHARM/80.502. 8-39. Ginevra. 1980.
24. Contreras-Moreno BZ, Rojas-V J, Méndez L, Celis MT. Preliminary Phytochemical screening of *Pimenta racemosa* var. racemosa (Myrtaceae) from Táchira-Venezuela. Pharmacology Online. 2014; 2: 61-68.
25. Weiss EA. Spice crops. CABI Publishing, USA. 2002.
26. Terpenoids Library Website. http://massfinder.com/wiki/Terpenoids_Library
27. Bendahou M, Muselli A, Grignon-Dubois M, Benyoucef M, Desjobert JM, Bernardini AF, Costa J. Antimicrobial activity and chemical composition of *Origanum glandulosum* Desf. essential oil and extract obtained by microwave extraction: comparison with hydrodistillation. Food Chem. 2008; 106: 132-139.
28. Tucker A, Maciarello M, Adams R, Landrum L, Zanoni T. Volatile leaf oils of Caribbean Myrtaceae. I. Three varieties of *Pimenta racemosa* (Miller) J. Moore of the Dominican Republic and the commercial bay oil. J Essent Oil Res. 1991; 3: 323-329.
29. Bello A, Rodriguez ML, Castineira N, Urquiola A, Rosado A, Pino JA. Chemical composition of the leaf oil of *Pimenta racemosa* (Mill.) J. Moore from Western Cuba. J Essent Oil Res. 1995; 7: 423-424.
30. Bello A, Urquiola A, García JJ, Rosado A, Pino JA. Essential oil from leaves of *Pimenta racemosa* (Mill) J. Moore (Myrtaceae) from Western Cuba. Ing Cienc Quim. 1998; 18: 21-23.
31. Jirovetz L, Buchbauer G, Stoilova I, Krastanov A, Stoyanova A, Schmidt E. Spice plants: chemical composition and antioxidant properties of *Pimenta* Lindl. essential oils. Part 2: *Pimenta racemosa* (Mill.) J.W. Moore leaf oil from Jamaica. Ernaehrung Nut. 2007; 7/8: 293-300.
32. Huelvas P, Mora F. Análisis y determinación de la actividad antibacteriana del aceite esencial de la *Pimenta racemosa* P. Miller (J.W. Moore) var. racemosa. Tésis de grado, Universidad de Los Andes, Facultad de Farmacia y Bioanálisis. 2009.
33. Alitonou GA, Noudogbessi JP, Sessou P, Tonouhewa A, Avlessi F, Menut C, Sohounhloue DCK. Chemical composition and biological activities of essential oils of *Pimenta racemosa* (Mill.) J. W. Moore. from Benin. Int J Bioscie. 2012; 2(9): 1-12.
34. Leyva M, Tacoronte J, Marquetti M. Composición química y efecto letal del aceite esencial de *Pimenta racemosa* (Myrtales: Myrtaceae) sobre *Blattella germanica* (Dictyoptera: Blattellidae). Rev Cubana Med Trop. 2007; 159: 2.

35. Contreras-Moreno B, Rojas J, Celis M, Rojas L, Mendez L, Landrum L. componentes volátiles de las hojas de *Pimenta racemosa* var. racemosa (Mill.) J.W. Moore (Myrtaceae) de Táchira - Venezuela. Bol Latinoam Caribe Plantas Medicin Aromat. 2014; 13(3): 305-310.

Medicinal plants and alkaloids in the fight against cancer

2

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ABSTRACT

Despite the successes achieved in the early diagnosis and treatment of some malignancies, cancer remains among the leading medical, scientific, social and economic challenges of the 21st century. The need to find a safe and highly effective cure for neoplastic diseases remains a major mission for modern science. Plants have been a source of medicinal substances for thousands of years, and phytoproducts continue to play an essential role in clinical practice. The proposed review presents data about mechanism of action, clinical application and toxicity of commercially available alkaloid-based antitumor agents (vinca alkaloids, taxanes, camptothecin's analogues) as well as information about some plant alkaloids (ellipticine, cepharanthine, liriodenine) with promising anticancer activity demonstrated in cell culture and animal model systems. Experimental results from preclinical investigations, putative cellular/molecular targets and mechanism(s) of action of these alkaloids are discussed.

Keywords: Alkaloids; Anticancer activity.

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1. INTRODUCTION

Alkaloids, originally described as cyclic nitrogen-containing biologically active substances, represent one of the largest and most diverse groups of secondary metabolites found in living organisms. Alkaloids have been traditionally isolated from higher plants but can be found also in animals and microorganisms. Because of their widely variable structure types, which exceed the boundaries of their traditional definition, these compounds were classified using different criteria such as: common natural source (e.g. a certain type of plants), similarity of the carbon skeleton or biochemical precursor. The most widely used classification is based on their biosynthetic pathways. Various alkaloid-containing medicinal plants have been used for centuries with therapeutic purposes by humans and ever since the isolation of morphine (the first isolated alkaloid) in 1806 - researchers have not stopped searching new alkaloids and studying their biological effects [1].

Some alkaloids are applied in modern medicine because of their relieving (ephedrine), analgesic (morphine) or anticancer (vinca alkaloids, paclitaxel and the other taxanes, analogues of camptothecin - irinotecan and topotecan) properties. Taxanes and vinca alkaloids (that inhibit cell spindle in a different manner) as well as irinocan and topotecan (the only FDA-approved topoisomerase I targeted inhibotors) are among the most potent anticancer drugs used in current clinical oncology [2].

2. VINCA ALKALOIDS

Vinca alkaloids (Fig. 1, Table 1) are isolated from the Madagascar pink inkle *Catharanthus roseus* G. Don (*Vinca rosea* L.) (Apocinaceae) [3]. *C. roseus* was used by various cultures for the treatment of diabetes, and vinblastine and vincristine were first discovered during an investigation of the plant as the source of potential oral hypoglycemic agents [4]. Vinca alkaloids were discovered in the 1950's by Canadian scientists, Robert Noble and Charles Beer for the first time. These compounds act on the mitotic phase of the cells by causing inhibition of microtubule dynamics leading to metaphase arrest and cell death. Although vinca alcaloids behave similarly to other anticancer agents such as colchicine and maytansine, there is no cross resistance between them [3]. Vinca alkaloids are the second most-used class of anticancer drugs [5]. There are four major vinca alkaloids in clinical use: vinblastine, vincristine, vinorelbine and vindesine [3].

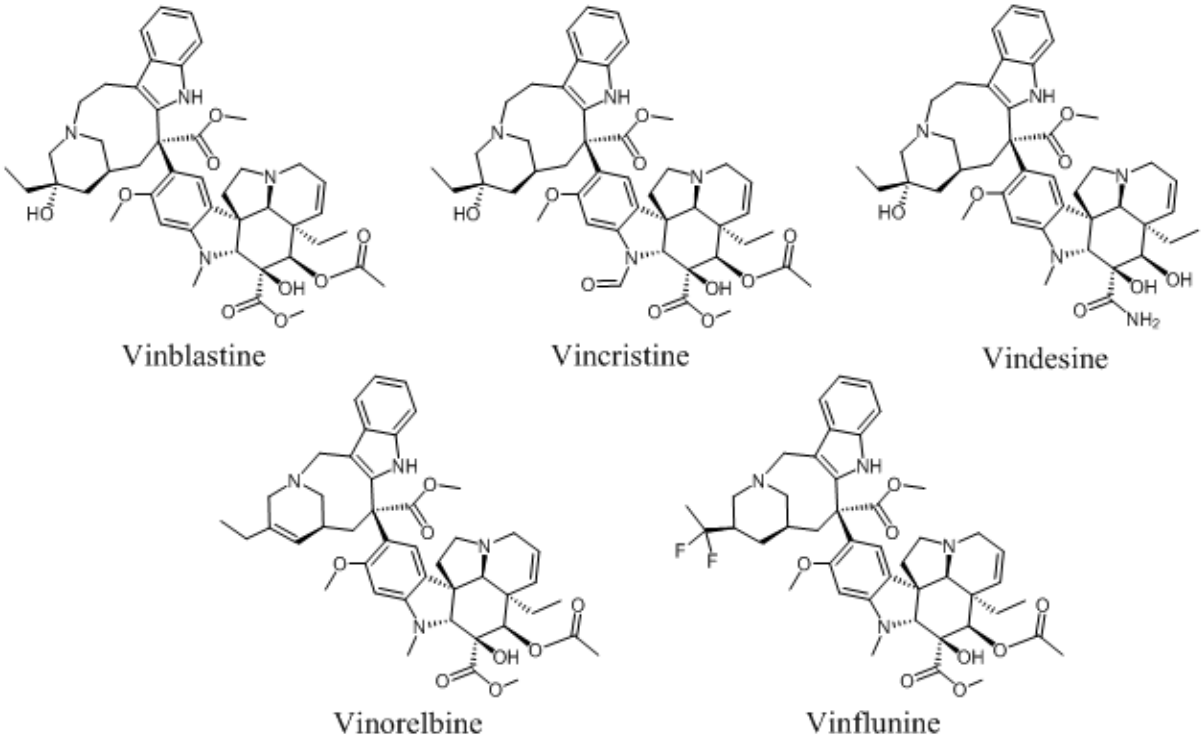

Figure 1. Vinca alkaloids

A new synthetic vinca alkaloid, vinflunine, was developed through the addition of two fluorine atoms. The drug is currently approved in Europe for clinical application in transitional cell carcinoma of the urothelium (TCCU) [6, 7].

Vinca alkaloids are widely used (usually included in combination chemotherapy regimens) in the treatment of hematological malignancies but are also effective against some solid tumors (breast cancer, testicular carcinoma, non-small cell lung cancer). They do not exhibit cross-resistance with drugs that alkylate deoxyribonucleic acid (DNA) and have a different mechanism of action [3]. Vincristine is also administered in the treatment of some non-malignant hematologic disorders such as thrombotic thrombocytopenia purpura [8].

Some vinca alkaloids are also available as oral formulations. For example, oral vinorelbine has demonstrated significant activity against non-small cell lung cancer (NSCLC) and has been reported to be advantageous in terms of cost savings compared to i.v. vinorelbine and other antineoplastic agents [9]. Vincristine sulfate liposomal injection has been approved in the USA by the Food and Drug Administration (FDA) for application in patients with Philadelphia chromosome (t(9;22)/BCR-ABL1) (Ph)-negative acute lymphoblastic leukemia [10].

Table 1. Vinca alkaloids - their medicinal application and toxicity

Vinca alkaloid	Medicinal application	Toxicity/side effects
Vinblastine	Hodgkin and non-Hodgkin lymphomas; breast cancer, germ cell tumors [11]	Toxicity to white blood cells, nausea, vomiting, constipation, dyspnea, chest or tumor pain, wheezing and fever [3]
Vincristine	Acute leukemia, rhabdomyosarcoma, neuroblastoma, Wilm's tumor, Hodgkin's disease and other lymphomas [3]	Peripheral neuropathy, myelosuppression, constipation, nervous system toxicity, nausea and vomiting [3, 11]
Vinorelbine	Non-small cell lung cancer, breast cancer [12, 13]	Decreased resistance to infection, bruising or bleeding, anemia, constipation, diarrhea, nausea, numbness or tingling in the hands and feet, peripheral neuropathy; Less common side-effects include alopecia and allergic reaction [3]
Vindesine	Acute lymphocytic leukemia, blast crisis of chronic myeloid leukemia, malignant melanoma, pediatric solid tumors and metastatic renal, breast, esophageal and colorectal carcinomas [14]	Neurologic (with paresthesias, without motor impairment), or hematologic (with leukopenia), sometimes alopecia, asthenia, muscle pains [15].
Vinflunine	Transitional cell carcinoma of the urothelium [7]	Neutropenia, anemia, constipation, fatigue [7]

Although, vinca alkaloids have quite similar chemical structures, their toxicologic profiles are different extensively [5]. Neutropenia is the principal dose-limiting toxicity of vinblastine, vindesine and vinorelbine. On the other hand, hematologic toxicity has been rarely reported for vincristine, severe myelosuppression has been observed in situations resulting in profoundly increased drug exposure and hepatic deficiency [3].

3. PACLITAXEL, CABAZITAXEL, DOCETAXEL (TAXANES)

3.1. Paclitaxel (Taxol)

Taxol (Fig. 2), a natural diterpene alkaloid, was extracted from the needles and bark of the Pacific yew tree *Taxus brevifolia (*Taxaceae), a small slow-growing evergreen, coniferous

tree. Its anticancer activity was reported for the first time in 1971 [16]. When it was commercially developed by the Bristol-Myers Squibb (BMS; New York, NY, USA) Taxol was renamed to paclitaxel [17].

Today Paclitaxel (Taxol) is one of the most effective broad-spectrum anticancer agents approved for the treatment of a wide range of neoplastic diseases including ovarian, breast, lung, head and neck cancers as well as Kaposi's sarcoma [18-22].

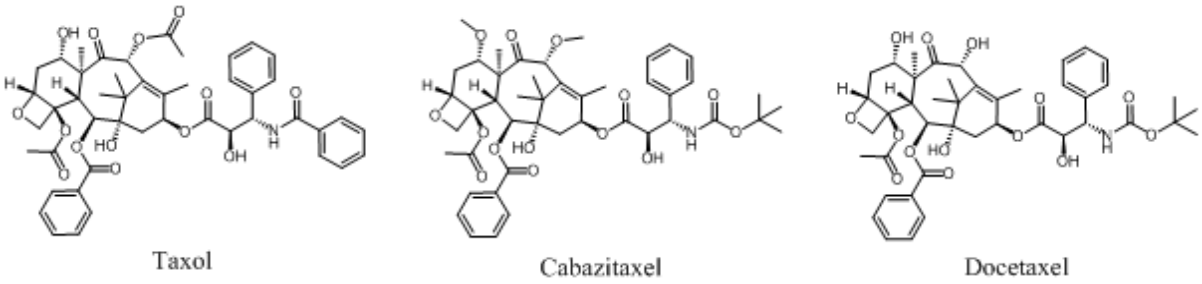

Figure 2. Taxanes

Paclitaxel is the prototype of the taxane family of antitumor drugs that have a unique mechanism of action. They stabilize GDP-bound tubulin in the microtubule (highly dynamic cellular polymers made of tubulin and associated proteins that are principal component of the cytoskeleton and play a key role during mitosis) resulting in arrest of the cell cycle progression through centrosomal impairment, induction of abnormal spindles and suppression of spindle microtubule dynamics and induction of apoptosis. For comparison, the other microtubule poisons (also known as spindle poisons or mitosis poisons), such as vinca alkaloids, colchicine, and cryptophycines, act in a different way by inhibiting tubulin polymerization. Taxanes are given intravenously and are eliminated via extensive hepatic metabolism and biliary excretion [23].

The following anticancer drugs belong to taxane family - classical taxanes (paclitaxel, docetaxel), the newer taxane cabazitaxel and the nanoparticle-bound nab-paclitaxel [23]. Taxanes represent one of the most powerful and widely used anticancer drugs in current clinical oncology [23, 24].

Although paclitaxel is effective for various cancer diseases, its application meets some obstacles:

i) Paclitaxel has extremely poor water solubility (<1 μg/ml) and does not sufficiently dissolve when administered in its crystalline form. It needs a relatively higher dose to

take effect compared to other anticancer drugs. The solvent used in the commercial formulation of solvent-based (sb)-paclitaxel is polyoxyethylated castor oil (Kolliphor® EL, formerly known as Cremophor EL; BASF SE, Ludwigshafen, Germany) [25, 26].

ii) The solvent Cremophor EL is associated with a high incidence of hypersensitivity reactions (HSRs). That is why the development of rapid drug desensitization has played an important role to allow patients with HSRs to taxanes to be safely re-treated. The mechanisms of these HSRs to taxol are not fully understood but there are data that Cremophor EL is responsible for complement activation. Some of these HSRs are suggested to be IgE-mediated [27-29].

iii) The therapeutic effect of this drug may be disturbed by acquired and intrinsic tumor resistance based on the role for ATP-binding cassette transporters (such as P-glycoprotein), tubulin isoforms, microtubule-associated proteins, tubulin gene mutations, and mitotic checkpoint signaling proteins [30]. A lot of efforts have been focused on the development of new approaches to overcome this problem [31-34]. To achieve a better therapeutic effect paclitaxel has also been delivered with microRNA [35], short hairpin RNA (shRNA) [36] and small interfering RNA (siRNA) molecules [37]. Currently there are no valid practical biomarkers that can predict resistance to the taxanes in (breast) cancer patients [38].

With regard to the toxicity the application of paclitaxel has been connected with risk of hypersensitivity reactions (that can be controlled with a premedication regimen consisting of corticosteroids and antihistamines); dose-limiting hematopoietic toxicity (specifically neutropenia), cumulative neurotoxicity (characterized principally by peripheral neurosensory manifestations), that are generally of mild to moderate severity, even in heavily pretreated patients. Additional adverse events associated with Taxol include arthralgia/myalgia, mucositis, alopecia [27, 39, 40].

Paclitaxel is not suitable for oral administration because of at least three reasons:

i) low water solubility;

ii) poor bioavailability, due to the affinity of paclitaxel for drug transporters highly expressed in the epithelial layer of the gastrointestinal tract, such as the drug efflux pump P-glycoprotein (PGP, ABCB1) and ABCC2 (cMOAT; MRP2);

iii) presystemic elimination by the cytochrome P450 (CYP) metabolic enzymes, especially CYP2C8 and CYP3A4, present in liver and gut wall, further hamper oral application of taxanes [25].

There are data that application of PGP and CYP3A4 blockers such as cyclosporine A and ritonavir can enhance the oral bioavailability of these anticancer drugs [25]. Other strategies recently used to improve the oral bioavailability of paclitaxel include the development of alternative formulations of paclitaxel on the basis of N-octyl-O-sulfate-chitosan micelles [41], 3,6-O,O'- dimyristoyl chitosan micelles [42], nanoemulsions [43], lipid nanocapsules [44], lipid nanosponges [45], etc.

Currently, two commercially available formulations of paclitaxel are Taxol and Abraxane. Nanoparticle albumin-bound paclitaxel (*nab*-paclitaxel; ABI 007 or Abraxane®; Celgene Inc, Odenton, MD), where paclitaxel is bound to albumin nano-particles (particle size of approximately 130 nanometers), is a solvent-free formulation of paclitaxel developed to reduce solvent-related adverse reactions and improve tumor penetration and efficacy via the physiologic transport properties of albumin [46].

Compared with sb-paclitaxel, *nab*-paclitaxel has several advantages, including: ability to deliver significantly higher doses of paclitaxel over a shorter infusion time (30 minutes vs 3 hours for sb-paclitaxel); decreased incidence of serious toxicities, including severe allergic reactions; enhanced transport of paclitaxel across endothelial cells and greater delivery of albumin/paclitaxel to tumors through receptor-mediated transport (transcytosis) [47, 48]. However, the preparation of Abraxane requires high-pressure homogenization of paclitaxel in the presence of human serum albumin, resulting in the high cost of the dosage form [49].

Nab-paclitaxel has been approved by Food and Drug Administration for the treatment of three solid tumors in the USA: metastatic breast cancer (in 2005); in combination with carboplatin for the first-line treatment of patients with locally advanced or metastatic NSCLC who are not candidates for curative surgery or radiation (2012); first-line treatment of patients with metastatic pancreatic cancer (2013). Nab-paclitaxel is also under investigation for the treatment of a number of other solid tumors [50].

3.2. Docetaxel

Docetaxel (Taxotere®) (Fig. 2) is a second generation taxane, a semi-synthetic analogue of paclitaxel approved by the FDA for the treatment of advanced breast cancer in 1996 [51], non-small-cell lung cancer (NSCLC) in 1999 [52], metastatic hormone-refractory prostate cancer (HRPC) in 2004, and head and neck cancer in 2006 [53]. The structure of paclitaxel and docetaxel differ in substitutions at the C-10 ring position and in the configuration of an ester side chain attached at C-13 (that is essential for antimicrotubule

activity) [54]. Because of its low aqueous solubility, docetaxel is solubilized by micelle formation with nonionic surfactant polysorbate 80 (Tween 80).

3.3. Cabazitaxel

Cabazitaxel (previously XRP-6258; trade name Jevtana®, Sanofi, UK) (Fig. 2) is a novel third-generation semisynthetic taxane, a dimethoxy derivative of docetaxel. It was approved by the FDA for the treatment of hormone-refractory prostate cancer in 2010. Cabazitaxel retains the taxane nuclear ring but has multiple side chain modifications that determine some of its advantages as compared to the other taxanes such as enhanced solubility in water-based solutions; decreased ability to induce multidrug resistance in cancer cells because of its lower affinity for the drug efflux pump, P-glycoprotein (ABCB1); better blood-brain barrier penetration confirmed after systemic administration in mouse models [54-56].

4. CAMPTOTHECIN AND ITS DERIVATIVES

4.1. Camptothecin

Camptothecin (Fig. 3) is a pentacyclic alkaloid isolated by Wall et al. [57] in the early 1960s from the wood, bark and fruit of the oriental tree *Camptotheca acuminate* (Chinese happy tree, Nyssaceae).

An effusion, injectables or powder, prepared from the tree, has been used in traditional Chinese medicine for treatment of various illnesses (common colds, psoriasis, liver and digestive problems), including tumors [58].

Camptothecin inhibits the action of the nuclear enzyme type-1 DNA topoisomerase by stabilizing the DNA-topoisomerase-1 cleavable complex formation that results in persisting single-stranded DNA breaks. During DNA replication, stabilized cleavage complexes convert into cytotoxic DNA double-strand breaks, ultimately causing cell death [59-62]. Its antineoplastic activity is limited by low stability and solubility as well as undesirable drug-drug interactions [62]. The initial clinical trials of sodium camptothecin were performed in the late 1960s and they revealed severe unpredictable toxicities and disappointing antitumor activity. The identification of its unique mechanism of action in the late 1970s and the

development of water soluble camptothecin analogs again provoke the interest in this class of compounds in the late 1980s [62, 63].

Three derivatives, topotecan [64], irinotecan [65] and belotecan [66] have been approved for the treatment of ovarian, small-cell lung, and refractory colorectal cancers.

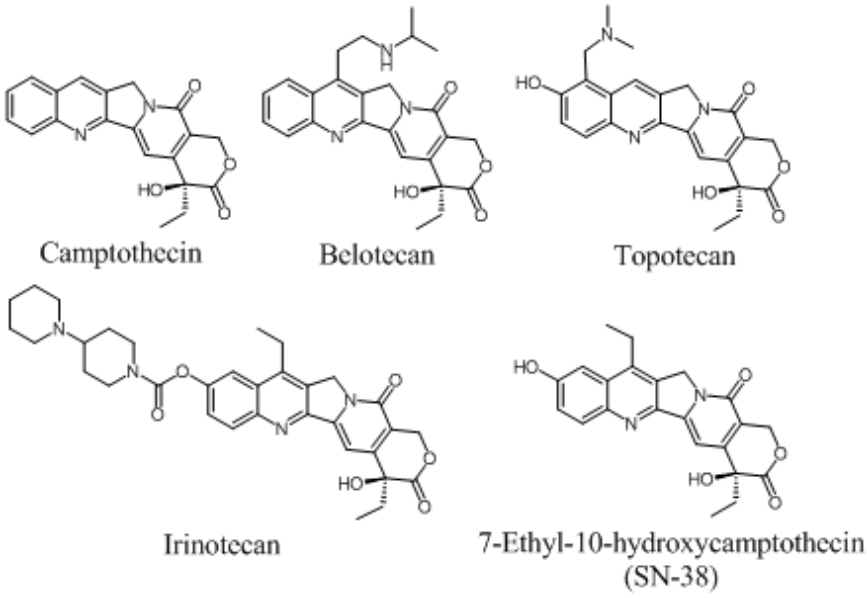

Figure 3. Camptothecin and its derivatives

4.2. Irinotecan

Irinotecan (Camptosar™) (Fig. 3) is a semi-synthetic derivative of camptothecin that shows some important advantages as compared to the parental structure, including higher aqueous solubility [67]; broad spectrum of antitumor activity both *in vitro* and *in vivo*; more predictable and clinically manageable toxicity [65].

Irinotecan hydrochloride was developed and approved in Japan for clinical use in small cell lung cancer, non-small cell lung cancer, cervical cancer and ovarian cancer in 1994. Subsequently, in France and the USA irinotecan was approved in 1995 and 1996, respectively, as a second-line chemotherapy in patients with recurrent or refractory metastatic colorectal cancer, following initial therapy with 5-flurouracil/leucovorin, and as a first-line chemotherapy in 1999 and 2000, respectively [68-70]. After the introduction of irinotecan into the management of metastatic colorectal cancer at the end of the last century, the survival

of patients has significantly improved [71]. Onivyde, a intravenous nanoliposomal irinotecan hydrochloride injection, was approved in 2015 by the FDA in combination with 5-fluorouracil and leucovorin for use in patients with advanced and metastatic pancreatic cancer refractory to gemcitabine based therapy [72, 73].

The two major dose-limiting toxicities of irinotecan are neutropenia and diarrhea [74, 75]. These adverse effects are observed in 13-25% of patients [76, 77].

The potent anti-tumor activity of irinotecan is due to rapid formation in the body of an in vivo active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38, Fig. 3) by carboxylesterase [71, 78]. SN-38 is several hundred fold more active than irinotecan.

Clinical use of SN38 is limited by its poor aqueous solubility and hydrolysis of the lactone ring at pH > 6 to inactive carboxylate form. A variety of drug delivery systems have been developed to improve the solubility and stability of SN38, and reduce its toxicity [79]. SN-38 is then metabolized in the liver to a non-toxic SN-38 glucuronide (SN-38G) [80], by uridine 5′-diphospho-glucuronosyltransferase 1A1 (*UGT1A1*). This metabolite is primarily excreted into the bile and transferred to the intestine [81, 82].

Polymorphism of the *UGT1A1* gene has been recognized to play an important role in irinotecan pharmacokinetics and severe toxicity [83]. Decreased UGT1A1 activity observed in *UGT1A1*28* and *UGT1A1*6* variants is associated with a decrease in the formation of SN-38G, the delayed metabolism of SN-38 and the occurrence of adverse events in irinotecan-based chemotherapy [84-88]. The allele frequency of *UGT1A1*28* has been documented to be lower in Asians (8.6–13.0 % in Japanese) than in Caucasians (29.5–38.8 %). In contrast, although the frequency of the *UGT1A1*6* allele is very low in Caucasians, it is relatively common in Asians (13.0–17.7 % in Japanese) [89]. In a recent meta-analysis, UGT1A1*6 polymorphisms were recognized as potential biomarkers, predicting irinotecan-induced severe toxicity in patients from Asia. Increased incidences of severe neutropenia have been found to occur in both high/medium and low doses of Irinotecan [90]. On the other hand, neither clinical response nor prognosis has been found to be significantly associated with the UGT1A1 gene polymorphisms [88]. Some UGT1A1 variants (such as UGT1A1*27) do not significantly predict severe irinotecan toxicity in cancer patients [91].

Pharmacogenetic factors other than *UGT1A1*, physiological factors, and environmental factors can also cause the large interindividual variability in SN-38 pharmacokinetics and contribute to irinotecan-induced toxicities [71].

4.3. Topotecan

Topotecan (Hycamtin) (Fig. 3) is approved as a second-line chemotherapy in patients with advanced and metastatic forms of small-cell lung cancer, ovarian cancer and cervical cancer and plays an important role in the treatment (in monotherapy or in combination with other cytotoxic agents) of these diseases [92-96]. Topotecan alone or in combination has been suggested to be active against retinoblastoma. It shows a favorable passage to the vitreous when given intravenously and intraarterially, and ocular toxicity is minimal by all routes of administration [97].

After GlaxoSmithKline received final FDA approval for Hycamtin Capsules in 2007, topotecan became the first topoisomerase I inhibitor for oral application [98].

The standard regimen of topotecan has generally mild nonhaematological toxicity and a well-defined haematological toxicity profile characterised by reversible and noncumulative neutropenia [94].

4.4. Belotecan

Belotecan (7-{2-(N-isopropylamino)ethyl}-(20S)-camptothecin, CKD602; Camtobell, Chong Keun Dang Corp., Seoul, Korea) (Fig. 3) is a synthetic derivative of camptothecin [99]. This compound possesses a water-solubilizing group at position 7 of the B-ring and higher water-solubility than the other camptothecin-derived anticancer drugs (>5 mg/kg). In addition, a hydroxyl group (-OH) was also introduced at position 20 of the E-ring, which enhances the anticancer activity of this agent [100].

The efficacy and safety of belotecan as a single agent and/or in combination with anticancer agents have been evaluated in clinical trials in patients with small cell lung cancer [101-104], ovarian cancer [105], carcinoma of the uterine cervix [106] with promising results reported in some of them.

The combination of cilengitide (the first anti-angiogenic small molecule drug candidate) [107] and belotecan has been demonstrated to express significant cytotoxicity and apoptosis in cultured human glioblastoma cells (U87MG, U251MG) and to reduce the volume of established U87MG tumors in nude mice [108]. Belotecan has been reported to show synergism with cisplatin for growth inhibitory effect on some human gastric cancer cell lines (SNU-5, SNU-16) [109].

The instability of the E-ring of camptothecins, along with their dose-limiting toxicity,

are the two main factors, limiting their effectiveness. The α-hydroxylactone opens through rapid hydrolysis (within minutes at physiological pH), forming the open ring carboxylate form, which binds tightly to serum albumin. Irinotecan and topotecan are administered intravenously, whereas oral formulations are not pursued, due to observed intestinal toxicity of irinotecan. The effectiveness of irinotecan and topotecan are also affected by drug efflux transporters (ABCG2) [110].

Many other water-insoluble (rubitecan, 9-aminocamptothecin, gimatecan, karenitecin, DB-67, etc.) and some water-soluble (exatecan, lurtotecan, sinotecan) analogues are currently under clinical evaluation [111]. There is also increasing interest in finding novel camptothecin analogues based on the already accumulated scientific data and elucidation of the mechanism of action of camptothecin as a topoisomerase I inhibitor [112].

5. CEPHARANTHINE

Cepharanthine (6',12'-Dimetoxy-2,2'-dimetyl-6,7-[metylenebis(oxy)]oxyacantan) (Fig. 4) is a naturally occurring biscoclaurine (bisbenzylisoquinoline) alkaloid extracted from the roots of Stephania cepharantha Hayata (Menispermaceae) [113], which is widely used, primarily in Japan, for the treatment of many acute and chronic diseases for more than 50 years.

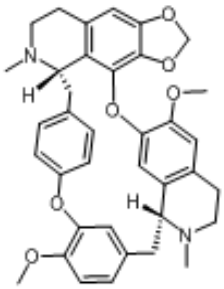

Figure 4. Cepharanthine

Among the conditions treated with cepharanthine are venomous snakebites, idiopathic thrombocytopenic purpura, xerostomia, sarcoidosis, refractory anemia, septic shock, exudative otitis media, etc. The alkaloid is approved by the Japanese Ministry of health for the treatment of radiation-caused leucopenia, alopecia areata and alopecia pityrodes [114, 115].

Various biological activities have been proved to be mediated by cepharanthine including: free-radical scavenging, membrane stabilizing, anti-inflamatory, antiallergic [114-118]. Cepharanthine has been reported to possess activity against various viruses including human immunodeficiency virus (HIV-1) [119-121], wide type and lamivudine-resistant hepatitis B virus (HBV) [122], severe acute respiratory syndrome coronavirus [123]. The antiplasmodial/antimalarial effect of cepharanthine (alone or in combination with antimalarial drugs) has also been demonstrated [124-125]. There are data that cepharanthine may be effective in the treatment of various types of ischemia-reperfusion injuries [126]. No safety issues have been observed with cepharanthine, and side effects are very rare reported [115].

The antitumor potential of the alkaloid deserves a special interest. There are data that cepharantine expresses anticancer properties in a number of ways including: cell cycle arrest [127, 128]; triggering apoptosis [127-131]; induction of autophagy cell death [128-132]; inhibition of angiogenesis [133]; prevention of metastasis [134, 135]; sensitization to anticancer drugs or radiation [136-140]; anti-inflammatory effects [114]; enhancement of endogenous immune response [115].

The cytotoxic/cytostatic activity of cepharanthine has been demonstrated in a wide variety of human cell lines established from some of the most common and/or invasive human cancers such as NSCLC (A549, H1299) [131], breast cancer (MCF-7, MDA-MB-231) [128], nasopharyngeal carcinoma [141], etc.

The therapeutic potential of cepharanthine against human cholangiocarcinoma (CCA) - an aggressive and highly metastatic tumor with poor responses to traditional chemotherapeutic agents, has been suggested. This alkaloids induces apoptosis (via caspase-3 and capase-9 activation) and significantly inhibits growth of human CCA cell lines in a concentration- and concentration-dependent manner, regardless of the histologic type of tumor origin; effectively reduces tumor size in CCA-inoculated mice without serious side effects; increases cell apoptosis in primary histocultures of CCA patients' tissues [130]. In addition, the anti-metastatic effects of cepharanthine on migration and invasion of human CCA cell lines KKU-M213 and KKU-M214 has been also demonstrated. This effect can be explained at least partially by the ability of cepharanthine to suppress function of intracellular adhesion molecule-1 (ICAM-1) and matrix metalloproteinase-2 (MMP-2) [135].

The ability of cepharanthine to inhibit the nuclear factor-kappaB (NF-kappaB) activity in cancer cells has been reported by different research groups [129, 130, 133]. Cepharanthine can suppress angiogenesis and growth of human oral squamous cell carcinoma cells in vitro and in vivo by inhibiting expression of vascular endothelial growth factor (VEGF) and

interleukin-8 (IL-8) involved in the blockade of NF-kappaB activity [133]. NF-kappaB has been suggested to be useful molecular target for treating primary effusion lymphoma (PEL) - non-Hodgkin's lymphoma caused by the Kaposi sarcoma-associated herpes virus - KSHV/HHV-8, that was originally identified in patients with HIV/AIDS, and cepharantine is recognized as a promising therapeutic agent for PEL [129].

Pretreatment with cepharanthine may reduce the renal toxicity of cisplatin through expression of metallothionein [142].

Cepharantine has been also reported to reverse multidrug resistance (MDR) by inhibiting the PGP expression and function in MDR cancer cells [143, 144].

6. ELLIPTICINE

Ellipticine (5,11-dimethyl-6H-pyrido[4,3-b]carbazole) (Fig. 5) is a pyridocarbazole alkaloid obtained from plant species (notably Aspidosperma, Tabernaemontana, Strychnos) of several genera of the Apocynaceae family [145].

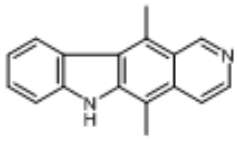

Figure 5. Ellipticine

Ellipticine is a DNA-damaging agent that functions as a prodrug. In the presence of cytochrome P450 (CYP1A, CYP3A and cytochrome b5) it is oxidized to 8-hydroxy-ellipticine which is more potent than the parent compound [146-148]. This alkaloid can induce the expression of these biotransformation enzymes in tumor tissues, thereby modulating its own metabolism, leading to its genotoxic and pharmacological effects [147-148].

Ellipticine is currently being actively investigated for its anticancer properties. The alkaloid and its derivatives have been found to express antitumor properties through multiple mechanisms involving cell cycle arrest and induction of cell death (apoptosis, autophagy) [145, 149]. Various DNA-mediated mechanisms of antitumor, mutagenic and cytotoxic

activities of ellipticine have been described: (i) intercalation into DNA, (ii) inhibition of DNA topoisomerase II activity, and (iii) covalent binding to DNA [149]. The antitumor activity has been suggested to be primarily mediated by the induction of DNA damage through the inhibition of topoisomerase II and formation of DNA adducts [150]. The ability to restore the transcription function of p53 tumor suppressor protein has been demonstrated [151]. Ellipticine has been shown to exhibit antitumor activity in various cell culture models including human breast cancer [147], endometrial cancer [150], neuroblastoma [152], T cell lymphoma [153]. There are data that it reduces the proliferation and self-renewal ability of aldehyde dehydrogenase 1 A1 (ALDH1A1)-positive breast cancer stem cells (BCSCs) and can be used in combination with paclitaxel for potential targeting of BCSCs [154]. The ability to inhibit telomerase activity in MDA-MB-231 breast cancer cell line extracts has been reported [155].

Various isoellipticines, isoellipticine derivatives and ellipticinium salts have been found to posess promising antitumor activities [156-158].

The highly active and less toxic derivative 2-N-Methyl-9-hydroxyellipticine acetate has been developed by Sanofi and is marketed in France under the trade name Celiptium for the treatment of breast cancer [146]. This drug seems to particularly improve the condition of patients suffering from oesteolytic breast cancer metastasis. Toxic side effects are nausea and vomiting (one-third of the patients), hypertension (less than 10% of the patients), muscular cramp (one-third of the patients), fatigue, which can be very pronounced (in most patients after 3 months of treatment), mouth dryness, and mycosis of the tongue and esophagus (less than 20% of the patients) [159].

7. LIRIODENINE

Liriodenine (8H-[1,3]benzodioxolo[6,5,4-de]benzo[g]quinolin-8-one) (Fig. 6) is a natural oxoaporphine alkaloid isolated for the first time from *Liriodendron tulipifera* L. (Magnoliaceae), and found subsequently in plant species of many genera [160], mainly in the families of Magnoliaceae, Annonaceae, Rutaceae, Monimiaceae, and Menispermaceae [161]. This compound is widely distributed and acts as a chemotaxonomic marker in the Annonaceae family [162].

Since 1975 various biological activities of liriodenine have been reported including its antiarrhythmic [163, 164], antiplatelet [165-167], antibacterial [168, 169], antifungal [169,

170] and mutagenic [171, 172] properties. Liriodenine has been reported to be active against *Leishmania donovani* and *Plasmodium falciparum* [173].

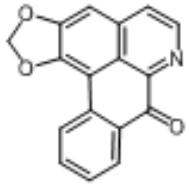

Figure 6. Liriodenine

The alkaloid regulates dopamine biosynthesis by partially reducing tyrosine hydroxylase activity (TH) and TH gene expression and has protective effects against L-DOPA-induced cytotoxicity in PC12 cells (Rat adrenal phaeochromocytoma cell line accepted as a suitable cell culture model for neurosecretion) [174]. The potential ability of liriodenine to reduce the motor dysfunctions in Parkinson's disease and its effect on the dopaminergic receptors have been recently discussed [175].

In addition, liriodenine has attracted attention for its antitumor activities. Liriodenine has been proved to exhibit cytotoxic effect in various types of human cell lines including A549 (NSCLC) [176]; SW480 (colon cancer) [177]; HepG2 and SK-Hep-1 (human liver cancer) [178]; CAOV-3 (ovarian cancer) [179], HEp-2 (laryngocarcinoma) [180]. The alkaloid exhibits moderate activity against four breast cancer cell lines - MCF-7, MCF-7 resistant to doxorubicin and two oestrogen receptor-deficient cell lines [181].

It has been suggested that several characteristics of the alkaloid contribute to its anticancer properties: i) Due to its planar structure, liridodenine is able to incorporate itself between adjacent base pairs of the DNA double helix molecule as an intercalating agent [182]; ii) Liriodenine has been shown to be a potent inhibitor of topoisomerase II enzyme (EC 5.99.1.3) both in vitro and in vivo [182]; iii) Ability to block cell cycle progression. It has been reported that liriodenine arrests cell cycle in G1 phase in HepG2 and SK-Hep-1 human liver cancer cell lines [178]; in S phase in CAOV human ovarian cancer cells [179]; in G1/S phase in SW480 human colon cancer cell line [177]; at the G2/M phase in A549 human NSCLC cells [176]; iv) Potential to induce apoptosis [176, 179, 180].

Nitric oxide (NO) production and p53 tumor suppressor gene expression are reported to be critical factors in liriodenine-induced inhibition in human wild-type p53 hepatoma cells

[178]. Increased intracellular NO production, protein levels of inducible NO synthase and p53 have been found in SW480 human colon cancer cells after treatment with liriodenine [177]. Upregulated levels of p53 were detected also in HEp-2 human laryngocarcinoma cells [180].

Due to the planar character and N-7/O-8 electron donor sites, liriodenine has the coordination capacity by forming chelates with several metal ions [161]. A platinum (II) complex of liriodenine has been found to induce cell cycle arrest in human BEL-7404 human hepatoma cells at both G2/M and S phase. It has been suggested that the nuclei, mitochondria and telomerase via G-quadruplex DNA stabilization all should be key targets for the antitumor mechanism of the complex, in which the central platinum (II) played a key role [183]. Zn(II), Co(II), Fe(II) and Mn(II) complexes with liriodenine bind more intensively to the DNA helix than does liriodenine [184]. It has been suggested that Liriodenine/Liriodenine metal complexes may offer a promising new approach for the development of higly effective and well tolerable anticancer agents [161]. However, the precise mechanism that underlies the antitumor action of liriodenine and its metal complexes remains to be elucidated.

8. CONCLUSION

From 1960 to 1980, about 35,000 plant species were investigated for antineoplastic activity by the National Cancer Institute of the National Institute of Health, USA [185], resulting in the discovery of paclitaxel and camptothecin, and the introduction of two powerful new groups of antitumor agents in clinical oncology (taxanes and camptothecin's analogues). Encouraging data from preclinical studies of diffrenet alkaloids and their derivatives (including cepharanthine, ellipticine and liriodenine) show that some of them may become a part of standard cancer treatment protocols. Thus, the investigations in this field are hihly promising and have to be continued.

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REFERENCES

1. Roberts MF, Wink M, eds. Alkaloids: Biochemistry, Ecology, and Medicinal

Applications. New York: Plenum Press; 1998.

2. Worden FP, Perissinotti AJ, Marini BL. Cancer Pharmacology and Pharmacotherapy review: study guide for oncology boards and MOC exams. New York: Demos Medical; 2016.
3. Kufe DW, Pollock RE, Weichselbaum RR, Bast RC, Gansler TS, Holland JF, et al. 6th ed. Hamilton (ON): BC Decker Inc; 2003. Holland-Frei cancer medicine.
4. Gidding CE, Kellie SJ, Kamps WA, de Graaf SS. Vincristine revisited. Crit Rev Oncol Hematol. 1999; 29(3): 267-287.
5. Moudi M, Go R, Yien CY, Nazre M. Vinca alkaloids. Int J Prev Med. 2013; 4(11): 1231-1235.
6. Bennouna J, Delord JP, Campone M, Nguyen L. Vinflunine: a new microtubule inhibitor agent. Clin Cancer Res. 2008; 14(6): 1625-1632.
7. Schutz FA, Bellmunt J, Rosenberg JE, Choueiri TK. Vinflunine: drug safety evaluation of this novel synthetic vinca alkaloid. Expert Opin Drug Saf. 2011; 10(4): 645-653.
8. Joly BS, Vanhoorelbeke K, Veyradier A. Understanding therapeutic targets in thrombotic thrombocytopenic purpura. Intensive Care Med. 2017.
9. Barletta G, Genova C, Rijavec E, Burrafato G, Biello F, Sini C, Dal Bello MG, Coco S, Truini A, Vanni I, Alama A, Beltramini S, Grassi MA, Boccardo F, Grossi F. Oral vinorelbine in the treatment of non-small-cell lung cancer. Expert Opin Pharmacother. 2014; 15(11): 1585-1599.
10. Raj TA, Smith AM, Moore AS. Vincristine sulfate liposomal injection for acute lymphoblastic leukemia. Int J Nanomedicine. 2013; 8: 4361-4369.
11. Rowinsky EK, Donehower RC. Paclitaxel (taxol). N Engl J Med. 1995; 332: 1004-1014.
12. Gregory RK, Smith IE. Vinorelbine - a clinical review. Br J Cancer. 2000; 82(12): 1907-1913.
13. Xu YC, Wang HX, Tang L, Ma Y, Zhang FC. A systematic review of vinorelbine for the treatment of breast cancer. Breast J. 2013; 19(2): 180-188.
14. Joel S. The comparative clinical pharmacology of vincristine and vindesine: does vindesine offer any advantage in clinical use? Cancer Treat Rev. 1996; 21(6): 513-525.
15. Bayssas M, Gouveia J, de Vassal F, Misset JL, Schwarzenberg L, Ribaud P, Musset M, Jasmin C, Hayat M, Mathé G. Vindesine: a new vinca alkaloid. Recent Results Cancer Res. 1980; 74: 91-97.
16. Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT. Plant antitumor agents. VI. Isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus*

brevifolia. J Am Chem Soc. 1971; 93: 2325-2327.

17. Goldspiel BR. Clinical overview of the taxanes. Pharmacotherapy. 1997; 17: 110S-125S.
18. Chu Q, Vincent M, Logan D, Mackay JA, Evans WK. Taxanes as first-line treatment for advanced non-small cell lung cancer: a systematic review and practice guideline. Lung Cancer. 2005; 50: 355-374.
19. Ozols RF. Systemic therapy for ovarian cancer: current status and new treatments. Semin Oncol. 2006; 33(2 Supl 6): S3-S11.
20. Estevez LG, Munoz M, Alvarez I, Fernandez Y, Garcia-Mata J, Ruiz-Borrego M, et al. Evidence-based use of taxanes in the adjuvant setting of breast cancer. A review of randomized phase III trials. Cancer Treat Rev. 2007; 33: 474-483.
21. Zhang Z, Mei L, Feng SS. Paclitaxel drug delivery systems. Expert Opin Drug Deliv. 2013; 10(3): 325-340.
22. Nehate C, Jain S, Saneja A, Khare V, Alam N, Dubey RD, Gupta PN. Paclitaxel formulations: challenges and novel delivery options. Curr Drug Deliv. 2014; 11(6): 666-686.
23. Joerger AC, Fersht AR. The p53 pathway: origins, inactivation in cancer, and emerging therapeutic approaches. Annu Rev Biochem. 2016; 85: 375-404.
24. Abal M, Andreu JM, Barasoain I. Taxanes: microtubule and centrosome targets, and cell cycle dependent mechanisms of action. Curr Cancer Drug Targets. 2003; 3(3): 193-203.
25. Jibodh RA, Lagas JS, Nuijen B, Beijnen JH, Schellens JH. Taxanes: old drugs, new oral formulations. Eur J Pharmacol. 2013; 717(1-3): 40-46.
26. Louage B, De Wever O, Hennink WE, De Geest BG. Developments and future clinical outlook of taxane nanomedicines. J Control Release. 2017; 253: 137-152.
27. Rowinsky EK, Eisenhauer EA, Chaudhry V, Arbuck SG, Donehower RC. Clinical toxicities encountered with paclitaxel (Taxol). Semin Oncol. 1993; 20(4 Suppl 3): 1-15.
28. Gelderblom H, Verweij J, Nooter K, Sparreboom A. Cremophor EL: the drawbacks and advantages of vehicle selection for drug formulation. Eur J Cancer. 2001; 37(13): 1590-1598.
29. Picard M, Castells MC. Re-visiting hypersensitivity reactions to taxanes: a comprehensive review. Clin Rev Allergy Immunol. 2015; 49(2): 177-191.
30. Chien AJ, Moasser MM. Cellular mechanisms of resistance to anthracyclines and taxanes in cancer: intrinsic and acquired. Semin Oncol. 2008; 35(2 Suppl 2): S1-S14; quiz S39.
31. Liu Y, Huang L, Liu F. Paclitaxel nanocrystals for overcoming multidrug resistance in cancer. Mol Pharm. 2010; 7(3): 863-869.

32. Bao Y, Guo Y, Zhuang X, Li D, Cheng B, Tan S, Zhang Z. D-α-tocopherol polyethylene glycol succinate-based redox-sensitive paclitaxel prodrug for overcoming multidrug resistance in cancer cells. Mol Pharm. 2014; 11(9): 3196-3209.

33. Jin X, Mo R, Ding Y, Zheng W, Zhang C. Paclitaxel-loaded N-octyl-O-sulfate chitosan micelles for superior cancer therapeutic efficacy and overcoming drug resistance. Mol Pharm. 2014; 11(1): 145-157.

34. Zhang F, Wang X, Xu X, Li M, Zhou J, Wang W. Reconstituted high density lipoprotein mediated targeted co-delivery of HZ08 and paclitaxel enhances the efficacy of paclitaxel in multidrug-resistant MCF-7 breast cancer cells. Eur J Pharm Sci. 2016; 92: 11-21.

35. Dai X, Fan W, Wang Y, Huang L, Jiang Y, Shi L, Mckinley D, Tan W, Tan C. Combined delivery of Let-7b microRNA and Paclitaxel via biodegradable nanoassemblies for the treatment of KRAS mutant cancer. Mol Pharm. 2016; 13: 520-533.

36. Shen J, Meng Q, Sui H, Yin Q, Zhang Z, Yu H, Li Y. IRGD conjugated TPGS mediates codelivery of paclitaxel and survivin shRNA for the reversal of lung cancer resistance. Mol Pharm. 2014; 11: 2579-2591.

37. Sun TM, Du JZ, Yao YD, Mao CQ, Dou S, Huang SY, Zhang PZ, Leong KW, Song EW, Wang J. Simultaneous delivery of siRNA and paclitaxel via a “Two-in-One” micelleplex promotes synergistic tumor suppression. ACS Nano. 2011; 5: 1483-1494.

38. Murray S, Briasoulis E, Linardou H, Bafaloukos D, Papadimitriou C. Taxane resistance in breast cancer: mechanisms, predictive biomarkers and circumvention strategies. Cancer Treat Rev. 2012; 38(7): 890-903.

39. Onetto N, Canetta R, Winograd B, Catane R, Dougan M, Grechko J, Burroughs J, Rozencweig M. Overview of taxol safety. J Natl Cancer Inst Monogr. 1993; (15): 131-139.

40. Morris PG, Fornier MN. Microtubule active agents: beyond the taxane frontier. Clin Cancer Res. 2008; 14(22): 7167-7172.

41. Mo R, Jin X, Li N, Ju C, Sun M, Zhang C, Ping Q. The mechanism of enhancement on oral absorption of paclitaxel by N-octyl-O-sulfate chitosan micelles. Biomaterials. 2011; 32(20): 4609-4620.

42. Silva DS, Almeida A, Prezotti F, Cury B, Campana-Filho SP, Sarmento B. Synthesis and characterization of 3,6-O,O'-dimyristoyl chitosan micelles for oral delivery of paclitaxel. Colloids Surf B Biointerfaces. 2017; 152: 220-228.

43. Choudhury H, Gorain B, Karmakar S, Biswas E, Dey G, Barik R, Mandal M, Pal TK. Improvement of cellular uptake, in vitro antitumor activity and sustained release profile

with increased bioavailability from a nanoemulsion platform. Int J Pharm. 2014; 460(1-2): 131-143.

44. Peltier S, Oger JM, Lagarce F, Couet W, Benoit JP. Enhanced oral paclitaxel bioavailability after administration of paclitaxel-loaded lipid nanocapsules. Pharm Res. 2006; 23: 1243-1250.

45. Torne SJ, Ansari KA, Vavia PR, Trotta F, Cavalli R. Enhanced oral paclitaxel bioavailability after administration of paclitaxel-loaded nanosponges. Drug Deliv. 2010; 17(6): 419-425.

46. Ibrahim NK, Desai N, Legha S, et al. Phase I and pharmacokinetic study of ABI-007, a Cremophor-free, protein-stabilized, nanoparticle formulation of paclitaxel. Clin Cancer Res. 2002; 8: 1038-1044.

47. Desai N, Trieu V, Yao Z, et al. Increased antitumor activity, intratumor paclitaxel concentrations, and endothelial cell transport of cremophor-free, albumin-bound paclitaxel, ABI-007, compared with cremophor-based paclitaxel. Clin Cancer Res. 2006; 12(4): 1317-1324.

48. Desai N, Trieu V, Damascelli B, Soon-Shiong P. SPARC expression correlates with tumor response to albumin-bound paclitaxel in head and neck cancer patients. Transl Oncol. 2009; 2(2): 59-64.

49. Hennenfent KL, Govindan R. Novel formulations of taxanes: a review. Old wine in a new bottle? Ann Oncol. 2006; 17(5): 735-749.

50. Kundranda MN, Niu J. Albumin-bound paclitaxel in solid tumors: clinical development and future directions. Drug Des Devel Ther. 2015; 9: 3767-3777.

51. Bissery MC, Nohynek G, Sanderink GJ, Lavelle F. Docetaxel (Taxotere): a review of preclinical and clinical experience. Part I: Preclinical experience. Anticancer Drugs. 1995; 6(3): 339-355, 363-368.

52. Guéritte F. General and recent aspects of the chemistry and structure-activity relationships of taxoids. Curr Pharm Des. 2001; 7(13): 1229-1249.

53. Ojima T, Nakamori M, Nakamura M, Katsuda M, Hayata K, Kato T, Kitadani J, Tabata H, Takeuchi A, Iwahashi M, Yamaue H. Neoadjuvant chemotherapy with divided-dose docetaxel, cisplatin and fluorouracil for patients with squamous cell carcinoma of the esophagus. Anticancer Res. 2016; 36(2): 829-834.

54. Chabner BA. The taxanes, vinca alkaloids, and their derivatives. In: Chabner BA, Longo DL. Harrisson's Manual of Oncology, 2nd Edition. 2014; McGraw-Hill Education.

55. Sémiond D, Sidhu SS, Bissery MC, Vrignaud P. Can taxanes provide benefit in patients with CNS tumors and in pediatric patients with tumors? An update on the preclinical

development of cabazitaxel. Cancer Chemother Pharmacol. 2013; 72(3): 515-528.

56. Tsao CK, Cutting E, Martin J, Oh WK. The role of cabazitaxel in the treatment of metastatic castration-resistant prostate cancer. Ther Adv Urol. 2014; 6(3): 97-104.

57. Wall ME, Wani MC, Cook CE, Palmer KH, McPhail AT, Sim GA. Plant antitumor agents I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from *Camptotheca acuminata*. J Am Chem Soc. 1966; 88: 3888-3890.

58. Huang KC. The Pharmacology of Chinese Herbs. Second Edition. CRC Press; 1999, 457-483.

59. Bailly C. Topoisomerase I poisons and suppressors as anticancer drugs. Curr Med Chem. 2000; 7: 39-58.

60. Liu LF, Duann P, Lin CT et al. Mechanism of action of camptothecin. Ann N Y Acad Sci. 1996; 803: 44-49.

61. Pommier Y, Pourquier P, Fan Y, Strumberg D. Mechanism of action of eukaryotic DNA topoisomerase I and drugs targeted to the enzyme. Biochim Biophys Acta. 1998; 1400: 83-105.

62. Martino E, Della Volpe S, Terribile E, Benetti E, Sakaj M, Centamore A, Sala A, Collina S. The long story of camptothecin: From traditional medicine to drugs. Bioorg Med Chem Lett. 2017; 27(4): 701-707.

63. Bomgaars L, Berg SL, Blaney SM. The development of camptothecin analogs in childhood cancers. Oncologist. 2001; 6(6): 506-516.

64. Houghton PJ, Cheshire PJ, Myers I, Stewart CF, Synold TW and Oughton JA. Evaluation of 9-dimethylaminomethyl-10-hydroxycamptothecin against xenografts derived from adult and chidhood solid tumors. Cancer Chemother Pharmacol. 1992; 31: 229-259.

65. Kunimoto T, Nitta K, Tanaka T, Uehara N, Baba H, Takeuchi M, Yokokura T, Sawada S, Miyasaka T, Mutai M. Antitumor activity of 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothec in, a novel water-soluble derivative of camptothecin, against murine tumors. Cancer Res. 1987; 47: 5944-5947.

66. Ahn SK, Choi NS, Jeong BS, Kim KK, Journ DJ, Kim JK, Lee SJ, Kim JW, Hong C, Jew S-S. Practical synthesis of (S)-7-(2-isopropylamino)ethylcamptothecin hydrochloride, potent topoisomerase I inhibitor. J Heterocycl Chem. 2000, 37: 1141-1144.

67. Gerrits CJ, de Jonge MJ, Schellens JH, Stoter G, Verweij J. Topoisomerase I inhibitors: the relevance of prolonged exposure for present clinical development. Br J Cancer. 1997; 76: 952-962.

68. U.S. FDA. FDA approves irinotecan as first-line therapy for colorectal cancer. Oncology (Williston Park). 2000; 14(5): 652, 654.

69. Tadokoro J, Kakihata K, Shimazaki M, Shiozawa T, Masatani S, Yamaguchi F, Sakata Y, Ariyoshi Y, Fukuoka M. Post-marketing surveillance (PMS) of all patients treated with irinotecan in Japan: clinical experience and ADR profile of 13,935 patients. Jpn J Clin Oncol. 2011; 41(9): 1101-1111.

70. Loupakis F, Cremolini C, Masi G, Lonardi S, Zagonel V, Salvatore L, Cortesi E, Tomasello G, Ronzoni M, Spadi R, Zaniboni A, Tonini G, Buonadonna A, Amoroso D, Chiara S, Carlomagno C, Boni C, Allegrini G, Boni L, Falcone A. Initial therapy with FOLFOXIRI and bevacizumab for metastatic colorectal cancer. N Engl J Med. 2014; 371(17): 1609-1618.

71. Fujita K, Kubota Y, Ishida H, Sasaki Y. Irinotecan, a key chemotherapeutic drug for metastatic colorectal cancer. World J Gastroenterol. 2015; 21(43): 12234-12248.

72. Passero FC Jr, Grapsa D, Syrigos KN, Saif MW. The safety and efficacy of Onivyde (irinotecan liposome injection) for the treatment of metastatic pancreatic cancer following gemcitabine-based therapy. Expert Rev Anticancer Ther. 2016; 16(7): 697-703.

73. Lamb YN, Scott LJ. Liposomal irinotecan: a review in metastatic pancreatic adenocarcinoma. Drugs. 2017; 77(7): 785-792.

74. Kudoh S, Fujiwara Y, Takada Y, Yamamoto H, Kinoshita A, Ariyoshi Y, Furuse K, Fukuoka M. Phase II study of irinotecan combined with cisplatin in patients with previously untreated small-cell lung cancer. West Japan Lung Cancer Group. J Clin Oncol. 1998; 16: 1068-1074.

75. Rothenberg ML, Meropol NJ, Poplin EA, Van Cutsem E, Wadler S. Mortality associated with irinotecan plus bolus fluorouracil/leucovorin: summary findings of an independent panel. J Clin Oncol. 2001; 19: 3801-3807.

76. Fukuoka M, Niitani H, Suzuki A, Motomiya M, Hasegawa K, Nishiwaki Y, Kuriyama T, Ariyoshi Y, Negoro S, Masuda N, et al. A phase II study of CPT-11, a new derivative of camptothecin, for previously untreated non-small-cell lung cancer. J Clin Oncol. 1992; 10(1): 16-20.

77. Shimada Y, Yoshino M, Wakui A, Nakao I, Futatsuki K, Sakata Y, Kambe M, Taguchi T, Ogawa N. Phase II study of CPT-11, a new camptothecin derivative, in metastatic colorectal cancer. CPT-11 Gastrointestinal Cancer Study Group. J Clin Oncol. 1993; 11(5): 909-913.

78. Ramesh M, Ahlawat P, Srinivas NR. Irinotecan and its active metabolite, SN-38: review of bioanalytical methods and recent update from clinical pharmacology perspectives. Biomed Chromatogr. 2010; 24(1): 104-123.

79. Palakurthi S. Challenges in SN38 drug delivery: current success and future directions. Expert Opin Drug Deliv. 2015; 12(12): 1911-1921.

80. Kawato Y, Aonuma M, Hirota Y, Kuga H, Sato K. Intracellular roles of SN-38, a metabolite of the camptothecin derivative CPT-11, in the antitumor effect of CPT-11. Cancer Res. 1991; 51(16): 4187-4191.

81. Rivory LP, Robert J. Identification and kinetics of a beta-glucuronide metabolite of SN-38 in human plasma after administration of the camptothecin derivative irinotecan. Cancer Chemother Pharmacol. 1995; 36: 176-179.

82. Haaz MC, Rivory L, Jantet S, Ratanasavanh D, Robert J. Glucuronidation of SN-38, the active metabolite of irinotecan, by human hepatic microsomes. Pharmacol Toxicol. 1997; 80(2): 91-96.

83. de Forni M, Bugat R, Chabot GG, Culine S, Extra JM, Gouyette A, Madelaine I, Marty ME, Mathieu-Boué A. Phase I and pharmacokinetic study of the camptothecin derivative irinotecan, administered on a weekly schedule in cancer patients. Cancer Res. 1994; 54(16): 4347-4354.

84. Hahn KK, Wolff JJ, Kolesar JM. Pharmacogenetics and irinotecan therapy. Am J Health Syst Pharm. 2006; 63: 2211-2217.

85. Innocenti F, Undevia SD, Iyer L, Chen PX, Das S, Kocherginsky M, Karrison T, Janisch L, Ramírez J, Rudin CM, Vokes EE, Ratain MJ. Genetic variants in the UDP-glucuronosyltransferase 1A1 gene predict the risk of severe neutropenia of irinotecan. J Clin Oncol. 2004; 22(8): 1382-1388.

86. Ando Y, Saka H, Ando M, Sawa T, Muro K, Ueoka H, Yokoyama A, Saitoh S, Shimokata K, Hasegawa Y. Polymorphisms of UDP-glucuronosyltransferase gene and irinotecan toxicity: a pharmacogenetic analysis. Cancer Res. 2000; 60(24): 6921-6926.

87. Han JY, Lim HS, Shin ES, Yoo YK, Park YH, Lee JE, Jang IJ, Lee DH, Lee JS. Comprehensive analysis of UGT1A polymorphisms predictive for pharmacokinetics and treatment outcome in patients with non-small-cell lung cancer treated with irinotecan and cisplatin. J Clin Oncol. 2006; 24(15): 2237-2244.

88. Xiao XG, Xia S, Zou M, Mei Q, Zhou L, Wang SJ, Chen Y. The relationship between UGT1A1 gene polymorphism and irinotecan effect on extensive-stage small-cell lung cancer. Onco Targets Ther. 2015; 8: 3575-3583.

89. Saito Y, Maekawa K, Ozawa S, Sawada J. Genetic polymorphisms and haplotypes of major drug metabolizing enzymes in East Asians and their comparison with other ethnic populations. Curr Pharmacogenom. 2007; 5: 49-78.

90. Cheng L, Li M, Hu J, Ren W, Xie L, Sun ZP, Liu BR, Xu GX, Dong XL, Qian XP. UGT1A1*6 polymorphisms are correlated with irinotecan-induced toxicity: a system review and meta-analysis in Asians. Cancer Chemother Pharmacol. 2014; 73(3): 551-560.

91. Fukuda M, Suetsugu T, Shimada M, Kitazaki T, Hashiguchi K, Kishimoto J, Harada T, Seto T, Ebi N, Takayama K, Sugio K, Semba H, Nakanishi Y, Ichinose Y. Prospective study of the UGT1A1*27 gene polymorphism during irinotecan therapy in patients with lung cancer: Results of Lung Oncology Group in Kyusyu (LOGIK1004B). Thorac Cancer. 2016; 7(4): 467-472.

92. Herzog TJ. Update on the role of topotecan in the treatment of recurrent ovarian cancer. Oncologist. 2002; 7 Suppl 5: 3-10.

93. Brave M, Dagher R, Farrell A, Abraham S, Ramchandani R, Gobburu J, Booth B, Jiang X, Sridhara R, Justice R, Pazdur R. Topotecan in combination with cisplatin for the treatment of stage IVB, recurrent, or persistent cervical cancer. Oncology (Williston Park). 2006; 20(11): 1401-1404, 1410; discussion 1410-1411, 1415-1416.

94. Garst J. Topotecan: an evolving option in the treatment of relapsed small cell lung cancer. Ther Clin Risk Manag. 2007; 3(6): 1087-1095.

95. Lorusso D, Pietragalla A, Mainenti S, Masciullo V, Di Vagno G, Scambia G. Review role of topotecan in gynaecological cancers: current indications and perspectives. Crit Rev Oncol Hematol. 2010; 74(3): 163-174.

96. Hartwell D, Jones J, Loveman E, Harris P, Clegg A, Bird A. Topotecan for relapsed small cell lung cancer: a systematic review and economic evaluation. Cancer Treat Rev. 2011; 37(3): 242-249.

97. Schaiquevich P, Carcaboso AM, Buitrago E, Taich P, Opezzo J, Bramuglia G, Chantada GL. Ocular pharmacology of topotecan and its activity in retinoblastoma. Retina. 2014; 34(9): 1719-1727.

98. U.S. FDA. Drug Approval Package for Hycamtin (topotecan) Capsules, 0.25 mg and 1 mg. Company: Smithkline Beecham Corporation. Approval Date: 10/11/2007. Available from: https://www.accessdata.fda.gov/drugsatfda_docs/nda/2007/020981_hycamtin_toc.cfm

99. Kim HK, Bang YJ, Heo DS, Shin SG, Kim NK. Phase I trial of CKD-602, a novel camptothecin derivative, in patients with advanced solid tumors. Proc Am Soc Clin Oncol. 2002; 21: abstr 393.

100. Lee JH, Lee JM, Kim JK, Ahn SK, Lee SJ, Kim MY, Jew SS, Park JG, Hong CI. Antitumor activity of 7-[2-(N-isopropylamino)ethyl]-(20S)-camptothecin, CKD602, as a potent DNA topoisomerase I inhibitor. Arch Pharm Res. 1998; 21(5): 581-590.

101. Kim GM, Kim YS, Ae Kang Y, Jeong JH, Kim SM, Hong YK, Sung JH, Lim ST, Kim JH, Kim SK, Cho BC. Efficacy and toxicity of belotecan for relapsed or refractory small cell lung cancer patients. J Thorac Oncol. 2012; 7(4): 731-736.

102. Hong J, Jung M, Kim YJ, Sym SJ, Kyung SY, Park J, Lee SP, Park JW, Cho EK, Jeong SH, Shin DB, Lee JH. Phase II study of combined belotecan and cisplatin as first-line chemotherapy in patients with extensive disease of small cell lung cancer. Cancer Chemother Pharmacol. 2012; 69(1): 215-220.

103. Yeo CD, Lee SH, Kim JS, Kim SJ, Kim SC, Kim YK, Kang HH, Yoon HK, Song JS, Moon HS, Kim JW, Kim KH, Shim BY, Kim CH. A multicenter phase II study of belotecan, a new camptothecin analogue, in elderly patients with previously untreated, extensive-stage small cell lung cancer. Cancer Chemother Pharmacol. 2013; 72(4): 809-814.

104. Lim S, Cho BC, Jung JY, Kim GM, Kim SH, Kim HR, Kim HS, Lim SM, Park JS, Lee JH, Kim D, Kim EY, Park MS, Kim YS, Kim SK, Chang J, Kim JH. Phase II study of camtobell inj. (belotecan) in combination with cisplatin in patients with previously untreated, extensive stage small cell lung cancer. Lung Cancer. 2013; 80(3): 313-318.

105. Choi CH, Lee YY, Song TJ, Park HS, Kim MK, Kim TJ, Lee JW, Lee JH, Bae DS, Kim BG. Phase II study of belotecan, a camptothecin analogue, in combination with carboplatin for the treatment of recurrent ovarian cancer. Cancer. 2011; 117(10): 2104-2111.

106. Hwang JH, Lim MC, Seo SS, Park SY, Kang S. Phase II study of belotecan (CKD 602) as a single agent in patients with recurrent or progressive carcinoma of uterine cervix. Jpn J Clin Oncol. 2011; 41(5): 624-629.

107. Mas-Moruno C, Rechenmacher F, Kessler H. Cilengitide: the first anti-angiogenic small molecule drug candidate design, synthesis and clinical evaluation. Anticancer Agents Med Chem. 2010; 10(10): 753-768.

108. Kim YH, Lee JK, Kim B, DeWitt JP, Lee JE, Han JH, Kim SK, Oh CW, Kim CY. Combination therapy of cilengitide with belotecan against experimental glioblastoma. Int J Cancer. 2013; 133(3): 749-756.

109. Jung JY, Song SH, Kim TY, Park JH, Jong HS, Im SA, Kim TY, Bang YJ, Kim NK. The synergism between Belotecan and cisplatin in gastric cancer. Cancer Res Treat. 2006; 38(3): 159-167.

110. Pommier Y. Drugging topoisomerases: lessons and challenges. ACS Chem Biol. 2013; 8(1): 82-95.

111. Liu YQ, Li WQ, Morris-Natschke SL, Qian K, Yang L, Zhu GX, Wu XB, Chen AL, Zhang SY, Nan X, Lee KH. Perspectives on biologically active camptothecin derivatives. Med Res Rev. 2015; 35(4): 753-789.

112. Dev S, Dhaneshwar SR, Mathew B. Discovery of camptothecin based topoisomerase i inhibitors: identification using an atom based 3D-QSAR, pharmacophore modeling, virtual screening and molecular docking approach. Comb Chem High Throughput Screen. 2016; 19(9): 752-763.

113. Kulka M. Bisbenzylisoquinoline alkaloids. In: The Alkaloids. Chemistry and Physiology. Ed. Manske R, Vol. 4. Academic Press, London, New York, 1965, 233.

114. Furusawa S, Wu J. The effects of biscoclaurine alkaloid cepharanthine on mammalian cells: implications for cancer, shock, and inflammatory diseases. Life Sci. 2007; 80(12): 1073-1079.

115. Rogosnitzky M, Danks R. Therapeutic potential of the biscoclaurine alkaloid, cepharanthine, for a range of clinical conditions. Pharmacol Rep. 2011; 63(2): 337-347.

116. Halicka D, Ita M, Tanaka T, Kurose A, Darzynkiewicz Z. Biscoclaurine alkaloid cepharanthine protects DNA in TK6 lymphoblastoid cells from constitutive oxidative damage. Pharmacol Rep. 2008; 60(1): 93-100.

117. Kudo K, Hagiwara S, Hasegawa A, Kusaka J, Koga H, Noguchi T. Cepharanthine exerts anti-inflammatory effects via NF-κB inhibition in a LPS-induced rat model of systemic inflammation. J Surg Res. 2011; 171(1): 199-204.

118. Huang H, Hu G, Wang C, Xu H, Chen X, Qian A. Cepharanthine, an alkaloid from Stephania cepharantha Hayata, inhibits the inflammatory response in the RAW264.7 cell and mouse models. Inflammation. 2014; 37(1): 235-246.

119. Okamoto M, Ono M, Baba M. Potent inhibition of HIV type 1 replication by an antiinflammatory alkaloid, cepharanthine, in chronically infected monocytic cells. AIDS Res Hum Retroviruses. 1998; 14(14): 1239-1245.

120. Okamoto M, Ono M, Baba M. Suppression of cytokine production and neural cell death by the anti-inflammatory alkaloid cepharanthine: a potential agent against HIV-1 encephalopathy. Biochem Pharmacol. 2001; 62(6): 747-753.

121. Matsuda K, Hattori S, Komizu Y, Kariya R, Ueoka R, Okada S. Cepharanthine inhibited HIV-1 cell-cell transmission and cell-free infection via modification of cell membrane fluidity. Bioorg Med Chem Lett. 2014; 24(9): 2115-2117.

122. Zhou YB, Wang YF, Zhang Y, Zheng LY, Yang XA, Wang N, Jiang JH, Ma F, Yin DT, Sun CY, Wang QD. In vitro activity of cepharanthine hydrochloride against clinical wild-

type and lamivudine-resistant hepatitis B virus isolates. Eur J Pharmacol. 2012; 683(1-3): 10-15.

123. Zhang CH, Wang YF, Liu XJ, Lu JH, Qian CW, Wan ZY, Yan XG, Zheng HY, Zhang MY, Xiong S, Li JX, Qi SY. Antiviral activity of cepharanthine against severe acute respiratory syndrome coronavirus in vitro. Chin Med J (Engl). 2005; 118(6): 493-496.

124. Desgrouas C, Chapus C, Desplans J, Travaille C, Pascual A, Baghdikian B, Ollivier E, Parzy D, Taudon N. In vitro antiplasmodial activity of cepharanthine. Malar J. 2014; 13: 327.

125. Desgrouas C, Dormoi J, Chapus C, Ollivier E, Parzy D, Taudon N. In vitro and in vivo combination of cepharanthine with anti-malarial drugs. Malar J. 2014; 13: 90.

126. Kusaka J, Hagiwara S, Hasegawa A, Kudo K, Koga H, Noguchi T. Cepharanthine improves renal ischemia-reperfusion injury in rats. J Surg Res. 2011; 171(1): 212-217.

127. Kikukawa Y, Okuno Y, Tatetsu H, Nakamura M, Harada N, Ueno S, Kamizaki Y, Mitsuya H, Hata H. Induction of cell cycle arrest and apoptosis in myeloma cells by cepharanthine, a biscoclaurine alkaloid. Int J Oncol. 2008; 33: 807-814.

128. Gao S, Li X, Ding X, Qi W, Yang Q. Cepharanthine induces autophagy, apoptosis and cell cycle arrest in breast cancer cells. Cell Physiol Biochem. 2017; 41(4): 1633-1648.

129. Takahashi-Makise N, Suzu S, Hiyoshi M, Ohsugi T, Katano H, Umezawa K, Okada S. Biscoclaurine alkaloid cepharanthine inhibits the growth of primary effusion lymphoma in vitro and in vivo and induces apoptosis via suppression of the NF-kappaB pathway. Int J Cancer. 2009; 125(6): 1464-1472.

130. Seubwai W, Vaeteewoottacharn K, Hiyoshi M, Suzu S, Puapairoj A, Wongkham C, Okada S, Wongkham S. Cepharanthine exerts antitumor activity on cholangiocarcinoma by inhibiting NF-kappaB. Cancer Sci. 2010; 101(7): 1590-1595.

131. Hua P, Sun M, Zhang G, Zhang Y, Tian X, Li X, Cui R, Zhang X. Cepharanthine induces apoptosis through reactive oxygen species and mitochondrial dysfunction in human non-small-cell lung cancer cells. Biochem Biophys Res Commun. 2015; 460(2): 136-142.

132. Law BY, Chan WK, Xu SW, Wang JR, Bai LP, Liu L, Wong VK. Natural small-molecule enhancers of autophagy induce autophagic cell death in apoptosis-defective cells. Sci Rep. 2014; 4: 5510.

133. Harada K, Ferdous T, Itashiki Y, Takii M, Mano T, Mori Y, Ueyama Y. Cepharanthine inhibits angiogenesis and tumorigenicity of human oral squamous cell carcinoma cells by suppressing expression of vascular endothelial growth factor and interleukin-8. Int J Oncol. 2009; 35: 1025-1035.

134. Okada K, Sakusabe N, Kobayashi A, Hoshi N, Sato K. Prevention of lung metastasis by intra-tumoral injection of Cepharanthin and staphylococcal enterotoxin B in transplantable rat osteosarcoma. Jpn J Cancer Res. 1999; 90: 928-933.

135. Uthaisar K, Seubwai W, Srikoon P, Vaeteewoottacharn K, Sawanyawisuth K, Okada S, Wongkham S. Cepharanthine suppresses metastatic potential of human cholangiocarcinoma cell lines. Asian Pac J Cancer Prev. 2012; 13 Suppl: 149-154.

136. Kono K, Takahashi JA, Ueba T, Mori H, Hashimoto N, Fukumoto M. Effects of combination chemotherapy with biscoclaurine-derived alkaloid (Cepharanthine) and nimustine hydrochloride on malignant glioma cell lines. J Neurooncol. 2002; 56: 101-108.

137. Nakajima A, Yamamoto Y, Taura K, Hata K, Fukumoto M, Uchinami H, Yonezawa K, Yamaoka Y. Beneficial effect of cepharanthine on overcoming drug-resistance of hepatocellular carcinoma. Int J Oncol. 2004; 24: 635-645.

138. Ikeda R, Che XF, Yamaguchi T, Ushiyama M, Zheng CL, Okumura H, Takeda Y, Shibayama Y, Nakamura K, Jeung HC, Furukawa T, Sumizawa T, Haraguchi M, Akiyama S, Yamada K. Cepharanthine potently enhances the sensitivity of anticancer agents in K562 cells. Cancer Sci. 2005; 96: 372-376.

139. Tamatani T, Azuma M, Motegi K, Takamaru N, Kawashima Y, Bando T. Cepharanthin-enhanced radiosensitivity through the inhibition of radiation-induced nuclear factor-kappaB activity in human oral squamous cell carcinoma cells. Int J Oncol. 2007; 31: 761-768.

140. Fang ZH, Li YJ, Chen Z, Wang JJ, Zhu LH. Inhibition of signal transducer and activator of transcription 3 and cyclooxygenase-2 is involved in radiosensitization of cepharanthine in HeLa cells. Int J Gynecol Cancer. 2013; 23(4): 608-614.

141. Liu G, Wu D, Liang X, Yue H, Cui Y. Mechanisms and in vitro effects of cepharanthine hydrochloride: Classification analysis of the drug-induced differentially-expressed genes of human nasopharyngeal carcinoma cells. Oncol Rep. 2015; 34(4): 2002-2010.

142. Sogawa N, Hirai K, Sogawa C, Ohyama K, Miyazaki I, Tsukamoto G, Asanuma M, Sasaki A, Kitayama S. Protective effect of cepharanthin on cisplatin-induced renal toxicity through metallothionein expression. Life Sci. 2013; 92(12): 727-732.

143. Enokida H, Gotanda T, Oku S, Imazono Y, Kubo H, Hanada T, Suzuki S, Inomata K, Kishiye T, Tahara Y, Nishiyama K, Nakagawa M. Reversal of P-glycoprotein-mediated paclitaxel resistance by new synthetic isoprenoids in human bladder cancer cell line. Jpn J Cancer Res. 2002; 93(9): 1037-1046.

144. Song YC, Xia W, Jiang JH, Wang QD. Reversal of multidrug resistance in drug-resistant cell line EAC/ADR by cepharanthine hydrochloride and its mechanism. Yaoxue Xuebao. 2005; 40(3): 204-207.

145. Stiborova M, Manhartova Z, Hodek P, Adam V, Kizek R, Eckschlager T, Frei E. Cytotoxicity of and DNA adduct formation by ellipticine and its micellar form in human leukemia cells in vitro. Neuro Endocrinol Lett. 2015; 36 Suppl 1: 22-28.

146. Butler MS, Newman DJ. Mother Nature's gifts to diseases of man: the impact of natural products on anti-infective, anticholestemics and anticancer drug discovery. Prog Drug Res. 2008; 65: 1, 3-44.

147. Stiborová M, Poljaková J, Martínková E, Bořek-Dohalská L, Eckschlager T, Kizek R, Frei E. Ellipticine cytotoxicity to cancer cell lines - a comparative study. Interdiscip Toxicol. 2011; 4(2): 98-105.

148. Stiborová M, Černá V, Moserová M, Mrízová I, Arlt VM, Frei E. The anticancer drug ellipticine activated with cytochrome P450 mediates DNA damage determining its pharmacological efficiencies: studies with rats, Hepatic Cytochrome P450 Reductase Null (HRN™) mice and pure enzymes. Int J Mol Sci. 2014; 16(1): 284-306.

149. Stiborová M, Frei E. Ellipticines as DNA-targeted chemotherapeutics. Curr Med Chem. 2014; 21(5): 575-591.

150. Kim JY, Lee SG, Chung JY, Kim YJ, Park JE, Koh H, Han MS, Park YC, Yoo YH, Kim JM. Ellipticine induces apoptosis in human endometrial cancer cells: the potential involvement of reactive oxygen species and mitogen-activated protein kinases. Toxicology. 2011; 289(2-3): 91-102.

151. Peng Y, Li C, Chen L, Sebti S, Chen J. Rescue of mutant p53 transcription function by ellipticine. Oncogene. 2003; 22(29): 4478-4487.

152. Poljaková J, Eckschlager T, Hrabeta J, Hrebacková J, Smutný S, Frei E, Martínek V, Kizek R, Stiborová M. The mechanism of cytotoxicity and DNA adduct formation by the anticancer drug ellipticine in human neuroblastoma cells. Biochem Pharmacol. 2009; 77(9): 1466-1479.

153. Savorani C, Manfé V, Biskup E, Gniadecki R. Ellipticine induces apoptosis in T-cell lymphoma via oxidative DNA damage. Leuk Lymphoma. 2015; 56(3): 739-747.

154. Pandrangi SL, Chikati R, Chauhan PS, Kumar CS, Banarji A, Saxena S. Effects of ellipticine on ALDH1A1-expressing breast cancer stem cells - an in vitro and in silico study. Tumour Biol. 2014; 35(1): 723-737.

155. Ghosh S, Kar A, Chowdhury S, Dasgupta D. Ellipticine binds to a human telomere sequence: an additional mode of action as a putative anticancer agent? Biochemistry. 2013; 52(24): 4127-4137.

156. Miller CM, O'Sullivan EC, Devine KJ, McCarthy FO. Synthesis and biological evaluation of novel isoellipticine derivatives and salts. Org Biomol Chem. 2012; 10(39): 7912-7921.

157. Deane FM, O'Sullivan EC, Maguire AR, Gilbert J, Sakoff JA, McCluskey A, McCarthy FO. Synthesis and evaluation of novel ellipticines as potential anti-cancer agents. Org Biomol Chem. 2013; 11(8): 1334-1344.

158. Russell EG, O'Sullivan EC, Miller CM, Stanicka J, McCarthy FO, Cotter TG. Ellipticine derivative induces potent cytostatic effect in acute myeloid leukaemia cells. Invest New Drugs. 2014; 32(6): 1113-1122.

159. Paoletti C, Le Pecq JB, Dat-Xuong N, Juret P, Garnier H, Amiel JL, Rouesse J. Antitumor activity, pharmacology, and toxicity of ellipticines, ellipticinium, and 9-hydroxy derivatives: preliminary clinical trials of 2-methyl-9-hydroxy ellipticinium (NSC 264-137). Recent Results Cancer Res. 1980; 74: 107-123.

160. Warthen D, Gooden EL, Jacobson M (1969). Tumor inhibitors: liriodenine, a cytotoxic alkaloid from *Annona glabra*. J Pharm Sci. 58(5): 637-638.

161. Chen ZF, Liu YC, Huang KB, Liang H. Alkaloid-metal based anticancer agents. Curr Top Med Chem. 2013; 13(17): 2104-2115.

162. Pinheiro MLB, Xavier CM., de Souza ADL, Rabelo DM, Batista CL, Batista RL, Costa EV., Campos FR, Barison A, Valdez RH., Ueda-Nakamura T, Nakamura, CV. Acanthoic acid and other constituents from the stem of *Annona amazonica* (Annonaceae). J Braz Chem Soc. 2009; 20: 1095-1102.

163. Chang GJ, Wu MH, Wu YC, Su MJ. Electrophysiological mechanisms for antiarrhythmic efficacy and positive inotropy of liriodenine, a natural aporphine alkaloid from *Fissistigma glaucescens*. Br J Pharmacol. 1996; 118: 1571-1583.

164. Chang KC, Su MJ, Peng YI, Shao CC, Wu YC, Tseng YZ. Mechanical effects of liriodenine on the left ventricular-arterial coupling in Wistar rats: pressure-stroke volume analysis. Br J Pharmacol. 2001; 133(1): 29-36.

165. Lin CH, Chang GJ, Su MJ, Wu YC, Teng CM, Ko FN. Pharmacological characteristics of liriodenine, isolated from *Fissistigma glaucescens*, a novel muscarinic receptor antagonist in guinea-pigs. Br J Pharmacol. 1994; 113(1): 275-281.

166. Chen KS, Ko FN, Teng CM, Wu YC. Antiplatelet and vasorelaxing actions of some aporphinoids. Planta Med. 1996; 62: 133-136.

167. Moharam BA, Jantan I, Jalil J, Shaari K. Inhibitory effects of phylligenin and quebrachitol isolated from *Mitrephora vulpina* on platelet activating factor receptor binding and platelet aggregation. Molecules. 2010; 15(11): 7840-7848.

168. Rahman MM, Lopa SS, Sadik G, et al. Antibacterial and cytotoxic compounds from the bark of *Cananga odorata*. Fitoterapia. 2005; 76: 758-761.

169. Costa EV, Pinheiro ML, Barison A, Campos FR, Salvador MJ, Maia BH, Cabral EC, Eberlin MN. Alkaloids from the bark of *Guatteria hispida* and their evaluation as antioxidant and antimicrobial agents. J Nat Prod. 2010; 73(6): 1180-1183.

170. Khan M, Kihara M, Omoloso A. Antimicrobial activity of *Michelia champaca*. Fitoterapia. 2002; 73: 744-748.

171. Nozaka T, Morimoto I, Ishino M, Natori S. Mutagenic principles in Sinomeni Caulis et Rhizoma. II. The mutagenicity of liriodenine in the basic fraction of the methanol extract. Chem Pharm Bull. (Tokyo). 1988; 36(6): 2259-2262.

172. Nozaka T, Watanabe F, Tadaki S, Ishino M, Morimoto I, Kunitomo J, Ishii H, Natori S. Mutagenicity of isoquinoline alkaloids, especially of the aporphine type. Mutat Res. 1990; 240(4): 267-279.

173. Camacho MR, Kirby GC, Warhurst DC, Croft SL, Phillipson JD. Oxoaporphine alkaloids and quinones from *Stephania dinklagei* and evaluation of their antiprotozoal activities. Planta Med. 2000; 66(5): 478-480.

174. Jin CM, Lee JJ, Yang YJ, Kim YM, Kim YK, Ryu SY, Lee MK. Liriodenine inhibits dopamine biosynthesis and L-DOPA-induced dopamine content in PC12 cells. Arch Pharm Res. 2007; 30(8): 984-990.

175. Fazel Nabavi S, Uriarte E, Rastrelli L, Modak B, Sobarzo-Sánchez E. Aporphines and parkinson's disease: medical tools for the future. Curr Top Med Chem. 2016; 16(17): 1906-1909.

176. Chang HC, Chang FR, Wu YC, Lai YH. Anti-cancer effect of liriodenine on human lung cancer cells. Kaohsiung J Med Sci. 2004; 20(8): 365-371.

177. Chen CY, Chen SY, Chen CH. Liriodenine induces G1/S cell cycle arrest in human colon cancer cells via nitric oxide-and p53-mediated pathway. Process Biochem. 2012; 47: 1460-1468.

178. Hsieh TJ, Liu TZ, Chern CL, Tsao DA, Lu FJ, Syu YH, Hsieh PY, Hu HS, Chang TT, Chen CH. Liriodenine inhibits the proliferation of human hepatoma cell lines by blocking cell cycle progression and nitric oxide-mediated activation of p53 expression. Food Chem Toxicol. 2005; 43(7): 1117-1126.

179. Nordin N, Majid NA, Hashim NM, Rahman MA, Hassan Z, Ali HM. Liriodenine, an aporphine alkaloid from *Enicosanthellum pulchrum*, inhibits proliferation of human ovarian cancer cells through induction of apoptosis via the mitochondrial signaling pathway and blocking cell cycle progression. Drug Des Devel Ther. 2015; 9: 1437-1448.

180. Li L, Xu Y, Wang B. Liriodenine induces the apoptosis of human laryngocarcinoma cells via the upregulation of p53 expression. Oncol Lett. 2015; 9(3): 1121-1127.

181. Khamis S, Bibby MC, Brown JE, Cooper PA, Scowen I, Wright CW. Phytochemistry and preliminary biological evaluation of *Cyathostemma argenteum*, a malaysian plant used traditionally for the treatment of breast cancer. Phytother Res. 2004; 18(7): 507-510.

182. Woo SH, Reynolds MC, Sun NJ, Cassady JM, Snapka RM. Inhibition of topoisomerase II by liriodenine. Biochem Pharmacol. 1997; 54(4): 467-473.

183. Li YL, Qin QP, Liu YC, Chen ZF, Liang H. A platinum(II) complex of liriodenine from traditional Chinese medicine (TCM): Cell cycle arrest, cell apoptosis induction and telomerase inhibition activity via G-quadruplex DNA stabilization. J Inorg Biochem. 2014; 137: 12-21.

184. Liu YC, Chen ZF, Liu LM, Peng Y, Hong X, Yang B, Liu HG, Liang H, Orvig C. Divalent later transition metal complexes of the traditional chinese medicine (TCM) liriodenine: coordination chemistry, cytotoxicity and DNA binding studies. Dalton Trans. 2009; 28(48): 10813-10823.

185. Katiyar C, Gupta A, Kanjilal S, Katiyar S. Drug discovery from plant sources: An integrated approach. Int Quart J Res Ayurveda. 2012; 33(1): 10-19.

Anticancer agent from medicinal plants: a review

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ABSTRACT

Cancer is a multistep process and characterized by irregular proliferation of cells. Usually these cells invade and destroy the normal cells, thus creating an imbalance in the body. Cancer is caused due to various factors like tobacco consumption, exposure of body to chemicals, dietary factors and environmental factors. Conventional medication for treatment of cancer has several impacts on healthy cells. There is also a problematic issue of increase in tumor resistance to the current therapeutic agents. Due to this, there is a great need to fight this disease with more effective medication. Natural product plays an important role in fight against cancer and provides a valuable gateway for the use and investigation of new therapeutic agents. Medicinal plants represent a good source of discovery and development of anticancer agents. Medicinal plants contain several biologically active compounds which enable them to cure cancer. They contain various secondary metabolites which include alkaloids, flavanoids, phenolics, carotenoids etc. Benefits of medicine from plant origin over synthetic (chemical) medicine have increased the importance of medicinal plants in the field of healthcare. Numerous medicinal plants are known to possess anticancer activity. Phytocompounds from these plant sources can prevent cancer initiation, promotion and progress by exerting anti-oxidant effects which mediates by the integration of NF-κB, Nrf2 and AP-1 signaling pathways. Overall this chapter provides a comprehensive repository for

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the scientific community working to develop new and improved medicines for cancer which poses serious threat to mankind all across the globe.

Keywords: Medicinal plants; Cancer; Phytocompounds; Anticancer activity.

1. INTRODUCTION

Cancer is an abnormal growth of cells in our bodies that can lead to death. It destroys the normal cells and creates and imbalance in the body. It is one of the most severe health problems in both developing and developed countries. It is very difficult to find the specific cause for cancer. However, tobacco use, alcohol consumption, environmental pollutant, infectious agents, custom habits and lifestyles are some commonly known reasons responsible for this disease.

Environmental factor that contributes to the cancer deaths includes tobacco, obesity, radiation, infection, heredity, stress, environmental pollutants and lack of physical activity (lifestyle, economic and behavioral factors). Smoking causes 90% of lung cancer [1]. It also causes kidney, stomach, pancreas, larynx, and bladder cancer [2]. Tobacco is responsible for about one in five cancer death cases globally [2].

Physical inactivity, obesity and diet are related to 30-35% of cancer deaths [3]. Physical inactivity is believed to contribute to the cancer risk. More than half of the effect from diet is due to the over nutrition. Some specific foods are related to the specific type of cancers like high salt diet causes gastric cancer, aflatoxin B1 causes liver cancer and chewing betel nut causes oral cancer [4].

Exposure to ionizing and non ionizing ultraviolet radiation causes cancer (up to 10%). Source of ionizing radiations includes radon gas and medical imaging; this radiation is not particularly a strong mutagen. When radiation combines with other cancer causing agents then it is more potent like radon with the tobacco smoke [5]. Prolonged ultraviolet ray exposure from sun light leads to skin cancer.

Hereditary also causes cancer in some cases, less that 0.3% population carries genetic mutations that cause cancer. Examples of cancer due to hereditary include inherited mutation in BRCA1 and BRCA2 genes, hereditary non-polyposis colorectal cancer. Some hormones also play an important role in the cancer development by promoting the cell proliferation [6].

Insulin like growth factors and their binding proteins play an important role in proliferation of cancerous cell [7].

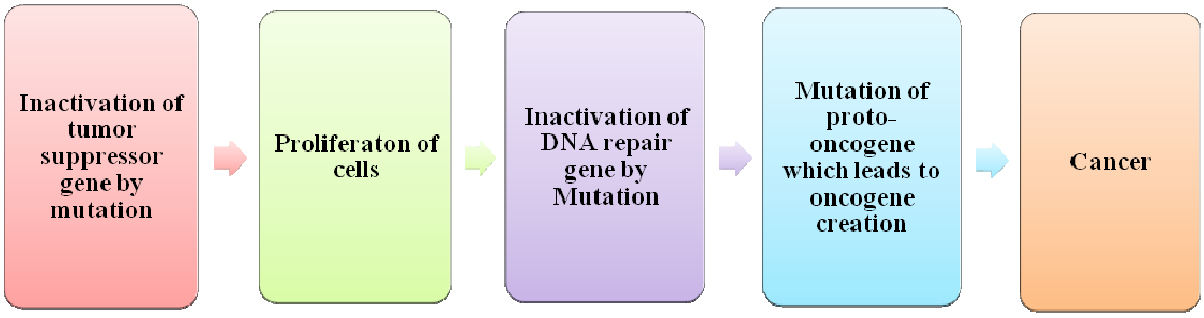

Figure 1. Development of cancer

'Staging' is a very important parameter in determining the severity of cancer. Depending on the stage of cancer, patients can be recommended drugs accordingly. There are four stages of cancer, each showing different properties and symptoms. These are tabulated as under:

STAGE 1	This is the primary stage of cancer with no visible symptoms. The tumour is not fully grown. A routine medical examination can help detect the presence of first stage cancer. If cancer is detected in this stage it would be easier to cure it.
STAGE 2	The tumour in this case is easily visible through scans. There are a few visible symptoms.
STAGE 3	The benign tumour is fully grown with detectable symptoms.
STAGE 4	This is the terminal stage cancer and no cure is possible in this stage. There is metastasis (spread) of tumour to distant parts of body. There are visible symptoms of cachexia (sudden significant weight loss), patches on skin in case of skin cancer.

There is an alternative approach to categorize cancer based on its severity and expansion as:

T	This is the first stage of cancer, and its severity depends on the spread of tumour from its original location.
N	Cancer is staged 'N', when the tumour spreads from its original location to lymphatic nodes.

M This is the stage where the tumour has metastasized to distant locations in the body and there is no possible cure for it.

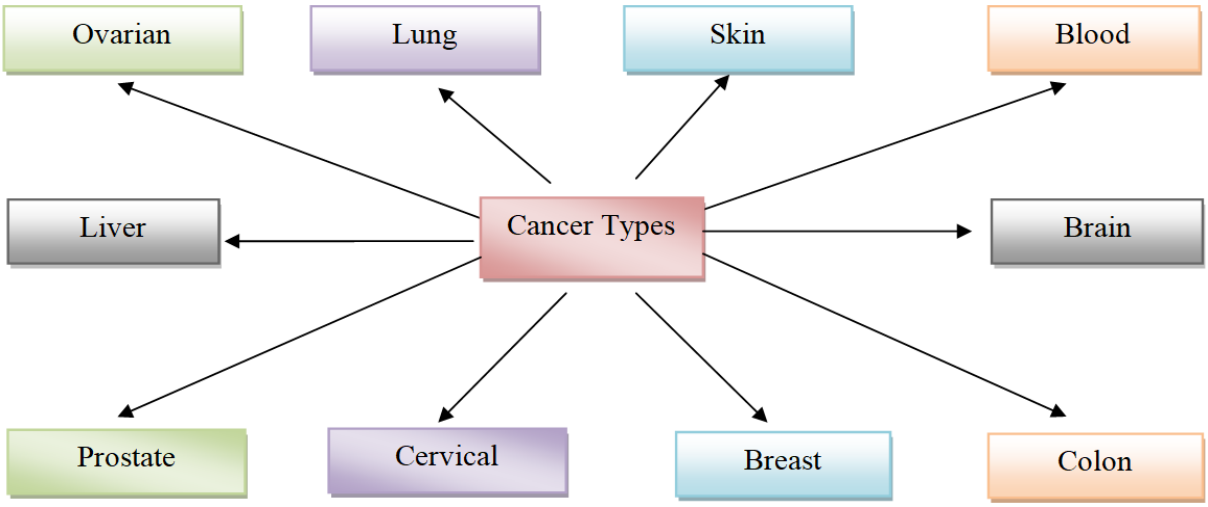

Figure 2. Types of cancer

The important preventive methods for most of the cancers include dietary changes, stopping the use of tobacco products, treating inflammatory diseases effectively, and taking nutritional supplements that aid immune functions. Current treatment for the cancer includes radiotherapy, chemotherapy and chemically derived drugs. Most commonly used cancer chemotherapy includes anti-metabolites, alkylating agents, platinum analogs and anti-tumor antibiotics. However, chemotherapy and radiotherapy puts patients under lots of strain and further causes health damage. Thus discovery of new anticancer agents from nature, especially plants is currently under investigation. Plants have always been a basis for the traditional medicine systems and they have provided continuous remedies to the mankind for thousands of years. Therapeutic potential of plants is based on the findings of thousands of years of use. First written records on hundreds of medicinal plants including opium and myrrh are listed on the clay tables [8]. Herbal medicines have been used for the centuries to treat various diseases. Herbal medicine uses plant extracts to treat the disease and promote the health of the patient. Aim of the herbal medicine is to restore the ability of body to protect, regulate and heal. Several modern drugs are made from the medicinal plants. Herbal products are taken in form of powder, paste, pills, liquid or raw material (extract). Use of certain herbal products tends to produce side effects and toxicities. In most cases, problem arises due to the

inappropriate use of the herbal products, mislabeling of plant materials, botanical misidentification, etc. This can be toxic when used for inappropriate indications or used in large amount or prepared inappropriately [9]. Therefore investigation on the herbal medicine efficacy is important to prevent from adverse effects. Thus, research has developed ways to find potential use of plant extracts for the treatment of cancer. Many plants have been already used to treat the various forms of cancer. Medicinal plants are considered as a repository of various bioactive compounds and they show wide range of biological activity which includes anti-tumor, anti-viral, anti-inflammatory and anti-malarial activity. Knowledge of the medicinal plants for the preparation of various drugs has been of great significance. Medicinal plants are considered as a rich source of wide variety of ingredients which can be used for the development of drug. Anticancer properties of several medicinal plants are used to find a lead compound that can block the development of cancer. Medicinal plant has various secondary metabolites such as terpenoids, flavanoids, alkaloid and steroids that have different pharmacological properties [10]. Medicinal plants such as *Allium sativum, Annona muricata, Berberies aristata, Catharanthus roseus, Linum usitatissimum, Podophyllum hexeandrum, Rubia cordifolia, Withania somnifera,* etc., show potential role in the inhibition of cancer cell proliferation. Therefore this chapter provides an overview of various medicinal plants and their major bioactive compounds utilized for treatment of cancer.

2. ANTICANCER ACTIVITY OF MEDICINAL PLANTS

2.1. *Allium sativum*

Allium sativum belongs to the Alliaceae family and native to the region between Mediterranean and China. It contains ajoene, allicin, alliin, allixin, γ-glutamyl-S-2-propenyl cysteine, diallyl disulfide, methyl allyl disulfide, S-allyl-cysteine and 1,2-vinyldiithin. Allicin shows antitumor activity in L5178Y lymphoma bearing mice. Methanolic extract of *A. sativum* (MEAS) shows anticancer activity against MCF7, A549 & DU145 and cell carcinoma of the bladder [11, 12].

2.2. *Annona muricata*

Annona muricata belongs to the Annonaceae family and commonly known as

Graviola. It contains acetogenins, β-sitosterol, stigmasterol, phenols, alkaloids, annoionoside, annoionol A, B, C, lycopene, lutein etc. It is distributed in the tropical regions of Central and South America, Western Africa and Southeast Asia. Acetogenins is the main compound and found in the leaves, seeds, bark and fruit of this plant. Acetogenis block the adenosine triphosphate production which inhibits the pump that removes cancer drug from the cell [13]. Acetogenins is identified to be toxic for various cancer cell lines such as pancreatic cancer, breast cancer, colonic adenocarcinoma, liver cancer and lymphoma [14].

2.3. *Astragalus membranaceus*

Astragalus membranaceus belongs to the Fabaceae family and is commonly known as Mongolian milkvetch. It contains astragloside, astraglan, calycosin, soyasapogenoside, quercein, kaempferol, etc. This plant is typically found in parts of China. It is used by the Chinese doctors to treat advanced cases of the liver cancer. A study reported that administration of this plant along with conventional treatment has shown higher survival rate of patients with advanced stage liver cancer as compared to the patients who were given conventional treatment. *Astragalus membranaceus* protects liver from toxic effects of chemotherapy [15]. Swainsonine which is an important compound of this plant is known to prevent metastases [16].

2.4. *Azadirachta indica*

Azadirachta indica belongs to the Meliaceae family and found in India and Indian subcontinent. It contains nimbin, nimbanene, nimbandiol, nimbolide, ascorbic acid, n-hexacosanol and amino acid, nimbiol, etc. It has been used for the treatment of skin cancer, buccal cancer, mammary cancer, prostate cancer and gastric cancer [17]. Ethanolic extract of *Azadirachta indica* causes prostate cancer cell death by apoptosis induction. It acts in a dose dependent manner and increase the fragmentation of DNA [18].

2.5. *Berberis aristata*

Berberis aristata belongs to the Berberidaceae family and commonly found in temperate and sub-tropical regions of Asia, Europe, and America. It is commonly known as Daruhaldi and roots of this plant contain berberine, berbamine, oxyxanthine,

jatrorhizine, epiberberine [19]. Methanolic extract of *B. aristata* showed potential anticancer activity against the human colon cancer cell line and inhibits HT29 cells in a concentration dependent manner [20]. It also significantly inhibits the carcinogenesis induced by 20-methylcholantherene in a dose dependent manner [21].

2.6. *Camellia sinensis*

Camellia sinensis belongs to the Theaceae family and is commonly known as green tea. It contains caffeine, theobromine, gallic acid, catechin, ampelopsin, epicatechin, etc. This plant is native to East Asia, the Indian Subcontinent and Southeast Asia but in today's time it is cultivated also in tropical and subtropical regions in the world. It contains polyphenolics possess anti-cancer and anti-mutagenic activity. Some evidence suggested that this plant has a protective effect against the colon and stomach cancer [22]. A study reported that green tea extract significantly inhibited the liver and leukemia tumor cells from DNA synthesis [16]. It also inhibits the cancer growth by eliminating the free radicals from the body.

2.7. *Cannabis sativa*

Cannabis sativa belongs to the Cannabinaceae family and native to the South Africa. It contains cannabinoids, cannabinol, anandamide, pinene, myrcene, etc. Cannabinoids shows potential inhibition towards cancer based on the experiments performed both in cultured cells and in animal models of cancer [23]. Cannabinoids present an interesting therapeutic potential such as antiemetics, analgesics, appetite stimulants in debilitating diseases, treatment of multiple sclerosis, spinal Tourette's syndrome, cord injuries, glaucoma and epilepsy [24]. Antineoplastic and proapoptotic properties of cannabinoids show emphasis effects of N-acylethanolamines [25]. Cannabinoids induces cancer cell death by apoptosis and inhibits proliferation of cancer cell [26].

2.8. *Catharanthus roseus*

Catharanthus roseus is an important medicinal plant and belongs to the Apocynaceae family. It is commonly known as Madagascar periwinkle. It contains actineoplastidemeric, vinblastin, vincrestine, vindesine, vindeline, tabersonine etc. Catharanthus is native to Madagascar but now-a-days it is being cultivated in Tanzania, Kenya, Kisi and many other

countries. This plant is used for the treatment of cancer, diabetes, fever and hypertension [27]. It contains many bioactive compounds which include vinblastine, vincristine, ajmalicine and serpentine [28]. Vinblastine and vincristin are commonly used for the treatment of leukemia and lymphoma [28].

2.9. *Curcuma longa*

Curcuma longa is commonly known as haldi in Hindi, harida in Sanskrit and turmeric in English. It is belongs to the Zingiberaceae family. This plant is native to Southern Asia and is also used as a coloring agent in Bangladeshi cuisine, Indian cuisine and also for many other purposes. It contains curcumin, curcuminoids, essential oil, turmerone, monoterpenes, diarylpentanoids, diterpenes, sesquiterpenes, triterpenoids, sterols, alkaloid, etc. Curcumin is the active ingredient of this plant, which is a polyphenol derived from plant rhizome and this plant is used for both cancer prevention and treatment. Curcumin shows protective effect by inhibiting the growth of several angiogenesis associates and tumor associated genes [29]. Curcumin possess anti-proliferative property by down regulating the numerous gene expressions which includes activator protein 1, NF-kappa B, cycloxygenase 2, epidermal growth receptor 1, nitric oxidase synthase and tumor necrosis factor [30].

2.10. *Glycine max*

Glycine max belongs to the Fabaceae family and native to East Asia. It is commonly known as Soya bean and rich in selenium, zinc, vitamins, isoflavones, amino acids, phytosterols and saponons. A study reported that soybean agglutinin inhibits the tumor growth in rats [31]. Isoflavones converts cancer cells to the normal by inducing cell differentiation. Genistein induce apoptosis in the cancer cells [16].

2. 11. *Linum usitatissimum*

Linum usitatissimum belongs to the Linaceae family and is rich in lignans. This short-lived perennial plant found in the western and southern Europe and western Asia. These plant lignans are converted to mammalian lignans (enterodiol and enterolactone) by bacterial fermentation in the colon [32] and they can then act as estrogens. Mammalian lignans appear to be anti-carcinogenic, lignan metabolites bear a structural similarity to estrogens and can

bind to estrogen receptors and inhibit the growth of estrogen-stimulated breast cancer [33]. Root extract of *Linum usitatissimum* induce significant amount inhibition of cell vitality and proliferation without performing the strong cytotoxicity in the human breast cell line MCF7 [34].

2.12. *Podophyllum hexeandrum*

Podophyllum hexeandrum belongs to the Podophyllaceae and found in Himalayan region. It is commonly known as Mayapple and contains podophyllotoxin, kaempferol, quercetin, asiragalin, essential oil, podophyllin. Podophyllotoxin has been used for the treatment of testicular and lung cancer as well as in certain leukemias [35]. Podophyllotoxin majorly found in the roots of this plant and used for the treatment of cancers, ulcers, wounds, constipation and tuberculosis [35].

2.13. *Oroxylum indicum*

Oroxylum indicum belongs to the Bignoniaceae family and is native to the Indian subcontinent. It contains baicalein, chrysin, oroxylin, scutellarein, pinostrobin, stigmast-7-en-3-ol. Several studies have shown the anticancer potential of this plant in various models. Ethanol extract (95%) of this plant showed cytotoxic effects against the Hep2 cell lines at 0.05% concentration [36]. Baicalein showed the antitumor effect on the human cancer cell lines and inhibits proliferation of HL-60 cell lines up to 50% at 25-30 µM concentration [37]. Aqueous and methanolic extract of *Oroxylum indicum* showed cytotxicity in selected tested cell lines and both the extracts exhibited moderate level of DNA protection against the oxidative stress [38].

2.14. *Punica granatum*

Punica granatum belongs to the Lythraceae family and fruit bearing deciduous shrub which is commonly known as pomegranate. It is originated in Iran and has been cultivated since ancient times throughout the Mediterranenan region and northern India. It is rich source of phenolic compounds, ellagitannins (ETs) and ellagic acid (EA) that metabolically convert to urolithins by the gut microbiota. Urolithins are found in high concentrations in colorectal cancer patients and urolithins inhibit proliferation of cancer cells and interfere with cell cycle

and induce apoptosis [39]. In a study, potential of pomegranate ellagitannins-derived compounds exhibited anti-proliferative and anti-aromatase activities in breast cancer cells [40].

2.15. *Rubia cordifolia*

Rubia cordifolia belongs to the Rubiaceae family and is commonly known as Indian madder. It contains cordifoliol, cordifodiol, anthraquinone, alizarin, etc. Mollugin showed anticancer activity against the lymphoid leukemia in mice and also inhibited the passive cutaneous anaphylaxis in rats [41]. Quinones and hexapeptides found in this plant showed the potential antitumor activity by 80S ribosome binding and this resulted in the inhibition of peptidyl tRNA and aminoacyl-tRNA binding which is required for the protein synthesis [42].

2.16. *Silybum marianum*

Silybum marianum belongs to the Asteraceae family and native to Southern Europe to Asia. It contains silymarin, silydianin, silychristin, silibinin, tyramine, histamine, essential oil, gamma linoleic acid, mucilage, etc. Silymarinis is the main compound of this plant which showed anticancer property against UV radiation induced skin cancer in mice and reduces the tumor multiplicity up to 78% [43]. Silymarins down regulates the gene product which is associated in the tumor cells proliferation, angiogensis, invasion and metastasis [44]. Silibinin also showed beneficial role in the human breast cancer [45]

2.17. *Taraxacum officinale*

Taraxacum officinale belongs to the Asteraceae family and is commonly known as Dandelions. It is a perennial plant and found in the temperate regions of the world such as Western, Central and Southern Europe, Northwest Africa, Northern Iran and Southwest Asia. It contains glycosides, terpenoids, carotenoids, choline, potassium salts, vitamin A, lecithin, etc. *Taraxacum officinale* decreased cell viability and increased the production of tumor necrosis factor α and interlukine-1α [46]. Leaves, root and flowers extracts of this plant was investigated against progression of tumor, among all the extracts leaf extract showed inhibition of breast cancer cell growth [47].

2.18. *Vernonia amygdalina*

Vernonia amygdalina belongs to the Compositae family and grows in tropical Africa. The bitterness in the plant extract is due factors such as glycoside, saponins, alkaloids and tannins. *Vernonia amygdalina* reverses the tumor in 106 MCF-7 breast cancer cells and also increased the basal apoptotic but decreased the angiogenic activity in mice [48]. *Vernonia amygdalina* shows anticancer activity in the human breast cancer cell lines (MCF-7 and MDA-MB-231) and inhibits the proliferation of cell lines in a dose and time dependent manner [49].

2.19. *Vismia laurentii*

Vismia laurentii belongs to the Guttiferae family and is found in the tropical and sub-tropical regions of world. It contains anthraquinones, xanthones and prenylated anthrones. It is used for the treatment of wounds and skin diseases. Xanthone V1 has shown anticancer potential against numerous cancer cell lines. It induces apoptosis and caspase-3 activity in CCRF-CEM cell line [50]. Fruits and seeds of this plant contain xanthones, naphtoquinones and anthraquinones which are thought to be responsible for the anticancer activity of this plant [51].

2.20. *Withania somnifera*

Withania somnifera is commonly known as ashwagandha in Hindi and Sanskrit, winter cherry in English. It belongs to the Solanaceae family and contains withanolides, withaferins, anferine, isopellertierine and sitoindosise. This plant grows majorly in India (especially Madhya Pradesh), Pakistan, Bangladesh, Sri Lanka and parts of Northern Africa. Due to its medicinal properties, leaves and roots have been used in the Indian traditional system of medicine and marketed globally. Extract of *Withania somnifera* modulates various biological responses [52]. It has been used in various preparations for its anti-stress, anti-peroxidative, anti-ageing, anti-inflammatory, anti-oxidant, anti-tumor, cardiotonic, and immunomodulatory properties [53]. Withanolide A and withaferin A is the main constitute of this plant. Withaferin A which is mostly present in the leaves produces rapid apoptosis in the cancer cells. Cell signaling pathways by this plant formulation largely depends up on the high content of withferin A present in it [53].

3. SOME OTHER MEDICINAL PLANTS WITH ANTI-CANCER ACTIVITY

Plant derived anticancer agents are effective inhibitors of cancer cells lines. Due to which there is a high demand of these plants for the production of therapeutically important compounds. There are several other medicinal plants all over the world, which are being used traditionally for cancer prevention and treatment. Some phytochemicals that are important active constituents of plants, including catechins, ursolic acid, silymarin, hecogenin glycyrrhizin, berberine, campothecin, gallic acid and various types of flavonoids, have shown promise in future cancer management. Some of the other medicinal plants that possess anticancer activity are discussed in the Table 1.

Table 1. List of other medicinal plants with anticancer activity

Plants	Family	Anti-cancer agents	References
Aegle marmelos	Rutaceae	Lupeol	[54]
Ailanthus altissima	Simaraubaceae	Ailantenol, ailnthanone	[55]
Andrographis paniculata	Acanthaceae	Andrographolide	[56]
Apium graveolens	Umbelliferae	Apigenin	[57]
Aloe ferox	Liliaceae	Aloe-emodin, emodin	[58, 59]
Alpinia galanga	Zingiberaceae	Pinocembrin	[54]
Ananas comosus	Bromeliaceae	Ananas bromelain	[60]
Aphanamixis polystachya	Meliaceae	Amooranin	[54]
Astragalus membranaceus	Papilionaceae	Swainsonine	[61]
Berberis vulgaris	Berberidaceae	Berberine	[54]
Betula utilis	Betulaceae	Betulin	[62]
Bleckeria vitensis	Apocynaceae	Ellipticine	[63]
Brucea antidysenterica	Simaraubaceae	Bruceantin	[63]
Campotheca acuminate	Nyssaceae	Campothecin	[63]
Chlorella pyrenoidosa	Oocystaceae	Lysine	[64]
Cephalotaxus harringtonia	Cephalotaxaceae	Homoharringtonine	[63]
Chelidonium jajus	Papaveraceae	Sanguinarine, chelerythrine, berberine	[65]
Colchicum luteum	Liliaceae	Colchicines, demecolcine	[66]

Plants	Family	Anti-cancer agents	References
Croton lechleri	Euphorbiaceae	Taspine	[67]
Diphylleia grayi	Berberidaceae	Diphyllin	[63]
Dysoxylum binectariferum	Meliaceae	Rohitukine	[63]
Echinops setifer	Asteraceae	Echinopsine	[18]
Erythronium americanum	Liliaceae	α-methylenebutyrolactone	[68]
Euphorbia semiperfoliata	Euphorbiaceae	Jatrophane	[67]
Fagopyrum esculentum	Polygonaceae	Rutin	[66]
Indigofera tinctoria	Leguminosae	Indirubins	[63]
Ginkgo biloba	Ginkoaceae	Ginkgolide	[69]
Glycyrrhiza glabra	Leguminosae	Glycyrrhizin	[70]
Gossypium barbadense	Malvaceae	Gossypol	[71]
Hydrastis canadensis	Ranunculaceae	Berberastine, candaline, hydrastine	[72]
Junchus effuses	Juncaceae	Tridecanone, juncanol, effusol, phenylpropanoid	[18]
Lantana camara	Verbenaceae	Isocamerine, camerine, lantanine, lantadene, micranine	[67]
Larrea tridentate	Zygophyllaceae	Terameprocol	[73]
Lentinus edodes	Agaricaceae	Lentinan	[74]
Lonicera japonica	Caprifoliaceae	Luteolin	[75]
Mappia foetida	Icacinaceae	Camptothecin	[76]
Newbouldia laevis	Bignoniaceae	2-acetylfuro-1,4-naphthoquinone	[51]
Nigella sativa	Ranunculaceae	Thymoquinone, dithymoquinone	[54]
Olea europea	Oleaceae	Oleic acid	[18]
Ocimum sanctum	Lamiaceae	Eugenol, orientin, vicenin	[54]
Oldenlandia diffusa	Rubiaceae	Ursolic acid	[54]
Paris polyphilla	Trilliaceae	Polyphyllin	[77]
Podophyllum hexandrum	Berberidaceae	Podophyllin	[78]
Plumbago zeylanica	Plumbaginaceae	Plumbagin	[79]
Prunella vulgaris	Labiatae	Oleanolic acid, ursolic acid	[54]
Psoralea corylifolia	Fabaceae	Bavachinin, psoralen, psoralidin	[54]

Plants	Family	Anti-cancer agents	References
Pteris multifida	Pteridaceae	Pterokaurane	[67]
Rubia cordifolia	Rubiaceae	Rubidianin, rubiadin, purpurin, xanthopurpurin	[54]
Scrophularia nodosa	Scrophulariaceae	Iridoid	[80]
Taxus brevifolia	Taxaceae	Taxanes, taxol cepholomannine	[81]
Zingiber officinale	Zingiberaceae	Curcumin, gingerenone A, gingeols, zingerone	[82]
Ziziphus nummularia	Rhamnaceae	Betulinic acid, betulin	[83]

4. CONCLUSION

Every year, cancer takes the life of millions of people. Various therapies are available for the cancer treatment but they have several limitations such as kidney damage, gastro-intestinal disorder, etc., due to which an alternative solution to this problem is required. Plant derived compounds possessing anti-cancerous activities have received huge amount of scientific attention. They play vital role in the cancer prevention and treatment. Pharmaceutical research has been done in countries like Germany, USA, Japan, France and China to improve the quality of herbal medicine for the cancer treatment [84]. Plants are the major source of secondary metabolites and an important source of pharmaceutical drugs. Herbal drug treatment is an ideal choice as it is comparatively cheaper and may be highly recommended to the poor and rural people for the effective treatment of cancer. Anticancer agents discovered from medicinal plants have played an important role in cancer treatment. It is documented that medicinal herbs have rich anticancer potential due to their immune-modulatory and antioxidant properties, and on the forefront whenever we talk about anticancer remedies, they are a significant source of synthetic and/or herbal origin. Bioactive compounds significantly influenced the cancer research on various aspects. Secondary metabolites from medicinal plants inhibits the DNA damage, arrest the cell cycle, inhibits the tumor cell angiogenesis and induce apoptosis thus prevents the cancer. Researchers must pay attention to the scientific rigor of studies of herbal drugs in the future to improve the status. Only few medicinal plants have been explored for their biological activity from 1000 species, so further investigations of plants in cancer treatment show a promising activity and it must

be taken into consideration. Therefore in this chapter selected plants have been explored for their biological activity and further more efforts are required to explore potent anticancer plants from nature to save humans life across the world from cancer.

REFERENCES

1. Biesalski HK, Bueno de Mesquita B, Chesson A, Chytil F, Grimble R, Hermus RJ, Köhrle J, Lotan R, Norpoth K, Pastorino U, Thurnham D. European Consensus Statement on Lung Cancer: risk factors and prevention. Lung Cancer Panel. Cancer J Clinic. 1998; 48(3): 167-176; discussion 164-166.
2. Kuper H, Boffetta P, Adami HO. Tobacco use and cancer causation: association by tumour type. J Int Med. 2002; 252(3): 206-224.
3. Kuper H, Adami HO, Boffetta P. Tobacco use, cancer causation and public health impact. J Int Med. 2002; 251(6): 455-466.
4. Kushi LH, Byers T, Doyle C, Bandera EV, McCullough M, McTiernan A, Gansler T, Andrews KS, Thun MJ. American Cancer Society Guidelines on Nutrition and Physical Activity for cancer prevention: reducing the risk of cancer with healthy food choices and physical activity. Cancer J Clinic. 2006; 56(5): 254-281.
5. Park S, Bae J, Nam BH, Yoo KY. Aetiology of cancer in Asia. Asian Pac J Cancer Prev. 2008; 9(3): 371-380.
6. Goubran HA, Kotb RR, Stakiw J, Emara ME, Burnouf T. Regulation of tumor growth and metastasis: the role of tumor microenvironment. Cancer Growth Metastasis. 2014; 7: 9-18.
7. Henderson BE, Bernstein L, Ross RK. Chapter 13: Hormones and the Etiology of Cancer. In: Bast RC, Kufe DW, Pollock RE, et al. Holland-Frei Cancer Medicine (5th ed.). Hamilton, Ontario: B.C. Decker. 2000.
8. Rowlands M-A, Gunnell D, Harris R, Vatten LJ, Holly JMP, Martin RM. Circulating insulin-like growth factor peptides and prostate cancer risk: a systematic review and meta-analysis. Int J Cancer. 2009; 124(10): 2416-2429.
9. Phua DH, Zosel A, Heard K. Dietary supplements and herbal medicine toxicities -when to anticipate them and how to manage them. Int J Emerg Med. 2009; 2(2): 69-76.
10. Sumner J. The natural history of medicinal plants. Timber Press. 2000.
11. Abdullaev FI. Plant derived agents against cancer. In: Gupta SK, editor. Pharmacology and therapeutics in the new millennium. Narosa publishing house: New Delhi, India 2001: 345-354.

12. Karmakar S, Choudhury SR, Banik NL, Swapan RK. Molecular mechanisms of anti-cancer action of garlic compounds in neuroblastoma. Anticancer Agents Med Chem. 2011; 11(4): 398-407.
13. Thomson M, Ali M. Garlic (*Allium sativum*): a review of its potential use as an anti-cancer agent. Curr Cancer Drug Targets. 2003; 3(1): 67-81.
14. Muriel J. M., Herbs or natural products that decrease cancer growth. Oncol Nursing Forum. 2004; 31(4): E75.
15. Prakash Om, Amit Kumar, Pawan Kumar, Ajeet. Anticancer potential of plants and natural products: a review. Am J Pharmacol Sci. 2013; 1: 104-115.
16. Wang J, Ito H, Shimura K. Enhancing effect of antitumor polysaccharide from Astralagus or Radix hedysarum onC3 cleavage production of macrophages in mice. Department of Pharmacology, Mie University School of Medicine, Japan. Mem Inst Oswaldo Cruz. 1991; 86(2): 159-164.
17. Sakarkar DM, Deshmukh VN. Ethnopharmacological review of traditional medicinal plants for anticancer activity. Int J Pharm Tech Res. 2011; 3: 298-308.
18. Pandey G, Madhuri S. Some medicinal plants as natural anticancer agents. Pharmacognosy Rev. 2009; 3: 259-263.
19. Kumar S, Suresh PK, Vijaybabu MR, Arunkumar A, Arunakaran J. Anticancer effects of ethanolic neem leaf extract on prostate cancer cell line (PC-3). J Ethnopharmacol. 2006; 105(12): 246-250.
20. Saied S, Batool S, Naz S. Phytochemical studies of *Berberis aristata*. J Basic Appl Sci. 2007; 3(1): 1-4.
21. Das S, Basu SP. Cytotoxic activity of methanolic extract of Berberis aristata DC on colon cancer. Global J Pharmacol. 2009; 3(3): 137-140.
22. Sharma K, Bairwa R, Chauhan N, Shrivastava B, Saini NK. Berberis aristata: a review. Int J Res Ayurveda Pharm. 2011; 2(2): 383-388.
23. Dreosti IE. Bioactive ingredients: antioxidants and polyphenols in tea. Nutr Rev. 1996; 54: S51-58.
24. Guzman M. Cannabinoids potential anticancer agents. Nature Rev Cancer. 2003; 3: 745-755.
25. Ben Amar M. Cannabinoids in medicine: a review of their therapeutic potential. J Ethnopharmacol. 2006; 105: 1-25.
26. Pushkarev VM, Kovzun OI, Tronko MD. Antineoplastic and apoptotic effects of cannabinoids. N-acylethanolamines. J Cancer Res Exp Oncol. 2008; 30(1): 6-21.

27. Velasco G, Sánchez C, Guzmán M. Towards the use of cannabinoids as antitumour agents. Nature Rev Cancer. 2012; 12(6): 436-444.
28. Don G. *Catharanthus roseus*. In: Ross IA, ed. Medicinal plants of the world: chemical constituents, traditional and modern uses. Vol 1. Totowa, New Jersey, USA: Humana Press; 2003: 175-196.
29. Van Der Heijden R, Jacobs DI, Snoeijer W, Hallard D, Verpoorte R. The *Catharanthus* alkaloids: pharmacognosy and biotechnology. Curr Med Chem. 2004; 11(5): 607-628.
30. Pratheeshkumar P, Sreekala C, Zhang Z, et al. Cancer prevention with promising natural products: mechanisms of action and molecular targets. Anti-Cancer Agents Med Chem. 2012; 12(10): 1159-1184.
31. Liener IE. Implications of antinutritional components in soybean foods. Crit Rev Food Sci Nutr. 1994; 34: 31-67.
32. Thompson LU, Robb P, Serraino M, Cheung F. Mammalian lignan production from various foods. Nutr Cancer. 1991; 16: 43-52.
33. Serraino M, Thompson LU. The effect of flaxseed supplementation on the initiation and promotional stages of mammary tumorigenesis. Nutr Cancer. 1992; 17: 153-948.
34. Szewczyk M, Abarzua S, Schlichting A, Nebe B, Piechulla B, Briese V, Richter DU. Effects of extracts from *Linum usitatissimum* on cell vitality, proliferation and cytotoxicity in human breast cancer cell lines. J Med Plants Res. 2014; 8(5): 237-245.
35. Imbert TF. Discovery of podophyllotoxins. Biochimie. 1998; 80: 207-222.
36. Sharma RK, Sharma S, Sharma SS. Seed germination behaviour of some medicinal plants of Lahaul and Spiti cold desert (Himachal Pradesh): implications for conservation and cultivation. Curr Sci. 2006; 90(8): 1113-1118.
37. Narisa K, Jenny MW, Heather MAC. Cytotoxic effect of four Thai edible plants on mammalian cell proliferation. Thai Pharmac Health Sci J. 2006; 1(3): 189.
38. Roy MK, Nakahara K, Na TV, Trakoontivakorn G, Takenaka M, Isobe S, et al. Baicalein, a flavonoid extracted from a methanolic extract of *Oroxylum indicum* inhibits proliferation of a cancer cell line in vitro via induction of apoptosis. J Archiv Pharmazie. 2007; 62(2): 149.
39. Kumar R.A., Rajkumar V., Guha G., Mathe L. Therapeutic potentials of *Oroxylum indicum* bark extracts. Chinese J Nat Med. 2010; 8(2): 121.
40. Adams LS, Zhang Y, Seeram, NP, Heber D, Chen S. Pomegranate ellagitannin-derived compounds exhibit antiproliferative and antiaromatase activity in breast cancer cells in vitro. Cancer Prev Res. 2010; 3: 108-113.

41. Devi PM, Siril EA. Traditional and modern use of Indian madder (*Rubia cordifolia* L.): an overview. Int J Pharmac Sci Rev Res. 2014; 25(1): 154-164.
42. Gupta PP, Srimal RC, Verma N, Tandon JS. Biological activity of *Rubia cordifolia* and isolation of an active principle. Pharmac Biol. 1999; 37(1): 46.
43. Itokawa H, Wang X, Lee KH. Homoharringtonine and related compounds, in anticancer agents from natural products. Cragg GM, Kingston DGI, Newman DJ, eds. Brunner-Routledge Psychology Press, Taylor & Francis Group, Boca Raton, Chapter 4, 2005: 47.
44. Agarwal R, Agarwal C, Ichikawa H, Singh RP, Agarwal BB. Anticancer potential of silymarin: from bench to bed side. Anticancer Res. 2006; 26(6B): 4457.
45. Kim S, Choi JH, Lim HI, Lee SK, Kim WW, Kim JS, et al. Silibinin prevents TPA-induced MMP-9 expression and VEGF secretion by inactivation of the Raf/MEK/ERK pathway in MCF-7 human breast cancer cells, Phytomed. 2009; 16(6-7): 573.
46. Li YL, Gan GP, Zhang HZ, Wu HZ, Li CL, Huang YP, Liu YW, Liu JW. A flavonoid glycoside isolated from *Smilax china* L. rhizome in vitro anticancer effects on human cancer cell lines. J Ethnopharmacol. 2007; 113(1): 115.
47. Hyun NK, Seung HH, Bong KS, Cheorl HK, Young HY, Hyung MK. *Taraxacum officinale* induces cytotoxicity through TNF-α and IL-1α secretion in Hep G2 cells. Life Sci. 2004; 74(9): 1149.
48. Sigstedt SC. Evaluation of aqueous extracts of *Taraxacum officinale* on growth and invasion of breast and prostate cancer cells. Int J Oncol. 2008; 32(5): 1085.
49. Lecia JG, Jetaime R, Ernest B I. *Vernonia amygdalina*: anticancer activity, authentication, and adultration detection. Int J Environ Res Public Health. 2008; 5(5): 342.
50. Wong FC, Woo CC, Hsu A, Tan BKH. The anti-cancer activities of vernonia amygdalina extract in human breast cancer cell lines are mediated through caspase-dependent and p53-independent pathways. PLoS One. 2013; 24: 8.
51. Kuete V, Wabo HK, Eyong KO, Feussi, MT, WienchB, Krusche B, et al. Anticancer activities of six selected natural compounds of some cameroonian medicinal plants. PLoS ONE. 2011; 6: 8.
52. Winters M. Ancient medicine, modern use: *Withania somnifera* and its potential role in integrative oncology. Altern Med Rev. 2006; 11(4): 269-277.
53. Alam S, Katiyar D, Goel R, Vats A, Mittal A. Role of herbals in cancer management. J Phytopharmacol. 2013; 2(6): 46-51.
54. Govind P. Some important anticancer herbs: a review, International Research Journal of Pharmacy, 2011, 2(7), 45-52

55. Chang M. Anticancer medicinal herbs. Human Science and Technology, Publishing House, Changsha, 1992.
56. Kumar RA, Sridevi K, Kumar NV, Nanduri S, Rajagopal S. Anticancer and immunostimulatory compounds from *Andrographis paniculata*. J Ethnopharmacol. 2004; 92(2-3): 291-295.
57. Sultana S, Ahmed S. Inhibitory effect of celery seeds extract on chemically induced hepatocarcinogenesis: modulation of cell proliferation, metabolism and altered hepatic foci development. Cancer Lett. 2005; 221: 11-20.
58. The effect of aloe-emodin on the proliferation of a new merkel carcinoma cell line. Am J Dermatopathol. 2002; 24(1): 17-22.
59. Pecere T, Gazzola MV, Micignat C. Aloe-emodin is a new type of anticancer agent with selective activity against neuro-ectodermal tumors. Cancer Res. 2000; 60: 2800-2804.
60. The wealth of India ‘a dictionary of Indian raw materials and industrial products. Vol. i (ab), 1985, p. 79.
61. The wealth of India ‘a dictionary of Indian raw materials and industrial products. Vol. i (ab), 1985, p. 109.
62. The wealth of India ‘a dictionary of Indian raw materials and industrial products. Vol. i (ab), 1985, p. 185.
63. Cragg GM, Newman DJ. Plants as a source of anti-cancer agents. J Ethnopharmacol. 2005; 100(1-2): 72-79.
64. Kantrajian HM, Talpaz M, Smith TL, Cortes J, Giles FJ. Homoharringtonine and low-dose cytarabine in the management of late chronic-phase chronic myelogenous leukemia. J Clin Oncol. 2000; 18: 3513-3521.
65. Senchina DS, Shah, Nisarg B, Busch MG. Effects of berberine, chelerythrine, and sanguinarine on proliferation in four human immortalized cell lines. J Iowa Acad Sci. 2012; 119: 1-4, 22-27.
66. Bruneton J. Pharmacognosy, phytochemisty medicinal plants. Lavoisier publisher, France, 1993, pp. 771-777.
67. Chavan SS, Damale MG, Shamkuwar PB, Pawar D. Traditional medicinal plants for anticancer activity. Int J Curr Pharmac Res. 2013; 5: 4.
68. Cavallito CJ, Haskell TH. Alpha-methylene butyrolactone from *Erythronium americanum*. J Am Chem Soc. 1946; 68(11): 2332-2334.
69. Tyler V. Herbs of choice. The therapeutic use of phytomedicinals. New York: Haworth press, 1994, pp. 32-33.
70. Ambasta SP, ed. The useful plants of India. 4th edn, National Institution Sci. Communication, Delhi, 2000, p. 239.

71. Ambasta SP, ed. The useful plants of India. 4th edn, National Institution Sci. Communication, Delhi, 2000, p. 243.
72. Brown PN, Roman MC. Determination of hydrastine and berberine in goldenseal raw materials, extracts, and dietary supplements by high-performance liquid chromatography with UV: collaborative study. J Aoac Int. 2008; 91(4): 694-701.
73. Prajapati ND, Purohit S, Sharma AK, Kumar T. Handbook of medicinal plants. Agrobios (India), 1st edn, 2003.
74. Ladanyi A, Timar J, Lapis K. Effect of lentinan on macrophage cytotoxicity against metastatic tumor cells. Cancer Immunol Immunother. 1993; 36: 123-126.
75. Mingji P. Cancer treatment with fu zheng pei ben principal. Fujjan Science and Technology publishing house, Fuzhou, 1992.
76. Desai AG, Qazi GN, Ganju RK, El-Tamer M, Singh J, Saxena AK, Bhat HK. Medicinal plants and cancer chemoprevention. Curr Drug Metab. 2008; 9(7): 581-591.
77. Henry SH, Bowers JC. Aflatoxin, hepatitis and worldwide liver cancer risks. Adv Exp Med Biol. 2002; 504: 229-233.
78. Nagar N, Jat RK, Saharan R, Verma S, Sharma D, Bansal K. Podophyllotoxin and their glycosidic derivatives. Pharmacophore. 2011; 2(2): 124-134.
79. Hazra B, Sarkar R, Bhattacharyya S, Ghosh PK, Chel G, Dinda B. Synthesis of plumbagin derivatives and their inhibitory activities against Ehrlich ascites carcinoma in vivo and *Leishmania donovani* promastigotes in vitro. Phytother Res. 2002; 16: 133-137.
80. Ardeshiry LA, Rezaie-Tavirani M, Mortazavi SA, Barzegar M, Moghadamnia SH, Rezaee MB. Study of anti cancer property of *Scrophularia striata* extract on the human astrocytoma cell line (1321). Iran J Pharmac Res. 2010; 9(4): 403-410.
81. Prota AE, Bargsten K, Zurwerra D, Field JJ, Díaz JF, Altmann K-H, et al. Molecular mechanism of action of microtubule-stabilizing anticancer agents. Science. 2013; 339: 587-590.
82. Katiyar SK, Agarwal R, Mukhtar H. Inhibition of tumor promotion in sencar mouse skin by ethanol extract of zingiber officinale rhizome. Cancer Res. 1996; 56(5): 1023-1030.
83. Sarek J, Kvasnica M, Urban MKJ, Hajduch M. Correlation of cytotoxic activity of betulinines and their hydroxy analogues. Bioorg Med Chem Lett. 2005; 15(19): 4196-4200.
84. Gunjan M, Naing TW, Saini RS, Ahmad AB, Naidu JR, Kumar I. Marketing trends & future prospects of herbal medicine in the treatment of various disease. World J Pharmac Res. 2015; 4(9): 132-155.

The most commonly used medicinal plants from Croatia

4

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ABSTRACT

In this chapter, most commonly used medicinal plants in complementary medicine in Croatia are reviewed: chamomile, lavender, chaste tree, black elder, hypericum, buckthorn, rosemary, and sage. These plants contain various biologically active substances such as flavonoids, phenolic acids, tannins, essential oils and anthraquinones which are responsible for different pharmacological activities such as anti-inflammatory, antibacterial, antifungal, hepatoprotective, etc. Description of each medicinal plant includes: botanical designation, medicinal drugs, origin, distribution, parts used for medicinal purpose with the chemical constituents responsible for specific pharmacological activity along with mechanism of action accompanied with chemical structure, pharmaceutical forms of drugs accompanied by dosage, side effects, contraindication and precaution, adulterants identifiers, possible substitutes, processing and information about available commercial products on national and international levels, where applicable. Information provided include summary of basic knowledge of the plant and is accompanied with recent researches in this field.

Keywords: Medicinal plants; Croatia; Chamomile; Lavender; Chaste tree; Black elder; *Hypericum*; Buckthorn; Rosemary; Sage.

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1. INTRODUCTION

In this chapter, the most characteristic medicinal plants from Croatia are described, namely: *Chamomilla recutita* (L.) Rauschert, *Lavandula officinalis* L., *Vitex agnus-castus* L., *Sambucus nigra* L., *Hypericum perforatum* L., *Rhamnus frangula* L., *Rosmarinus officinalis* L. and *Salvia officinalis* L. These plants contain various biologically active substances such as flavonoids, phenolic acids, tannins, essential oils and anthraquinones which are responsible for different pharmacological activities such as antiinflammatory, antibacterial, antifungal, hepatoprotective, etc. Descriptions include: botanical designation, photographs of plants and drugs, origin, distribution, parts used for medicinal purpose with the chemical constituents responsible for specific pharmacological activity. These are accompanied with a mechanism of action and related to chemical constituents. Application and appropriate pharmaceutical forms of drugs are suggested. These are followed by side effects, contraindication and precaution. Possible adulterants and substitutes that can be used erroneously or as a replacement, respectively, are stated. If data is available, information on processing and available commercial products on national and international levels is given.

According to the information provided by Croatian Central Bureau of Statistics in year 2007, 2207 tones of medicinal and aromatic plants were produced on the total surface of about 2200 hectares. The most cultivated medicinal plants are chamomile (88% of total surface) and lavender (11%). These plants as well as chaste tree, black elder, St John's Wort, buckthorn, rosemary and sage, on which we have worked in the last decade were selected as major representatives of Croatian medicinal plants, due to high content of biologically active substances. Information provided include summary of basic knowledge of the plant and is accompanied with recent researches in this field.

2. *Chamomilla recutita*

Chamomilla recutita (L.) Rauschert (Fig. 1) is found under these common names: chamomile, camomile, camamilla, camomilla, chamomille, Commune (Eng.), Kamille, Kamillen (Ger.), fleurs de petit camomille, matricaire (Fr.), manzanilla (Sp.). It is a 10-30 cm tall herbaceous annual with erect, branching stems and alternate, divided leaves. The capitulum comprises 12-20 white ligulate flowers surrounding a conical hollow receptacle on which numerous tubular florets are inserted. The fruits are small, smooth, yellowish. The

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herbal drug, *Chamomillae flos* (Fig. 1), consists of the dried flowering heads of *Chamomilla recutita* (L.) Rauschert (synonyms: *Matricaria chamomila* L., *M. recutita* L.), Asteraceae [1, 2]. The flowers are separated from plant and on basis of length of pedicle are classified in I and II class used as row material and third class is used for water distillation of essential oil.

The plant is indigenous to northern Europe and grows wild in central European countries; it is especially abundant in Eastern Europe. This species is also found in western Asia, the Mediterranean region of northern Africa and the United States of America. It is cultivated in many countries because of the great interest of pharmaceutical, cosmetic and food industries.

Figure 1. *Chamomilla recutita*

Flower heads are used for medicinal purpose. They are gathered when plants are in full blossom and dried. The drug *Chamomillae flos* or *Matricariae flos* is official in many pharmacopoeias. It contains an essential oil, which has an intense blue color owing to its chamazulene content. Other major constituents of the oil include bisabolols and its oxides and en-in-dicycloethers. The drug also contains flavonoids (apigenin and related flavonoid glycosides) and coumarins. Chemical structures of constituents are presented in Fig. 2 [3, 4].

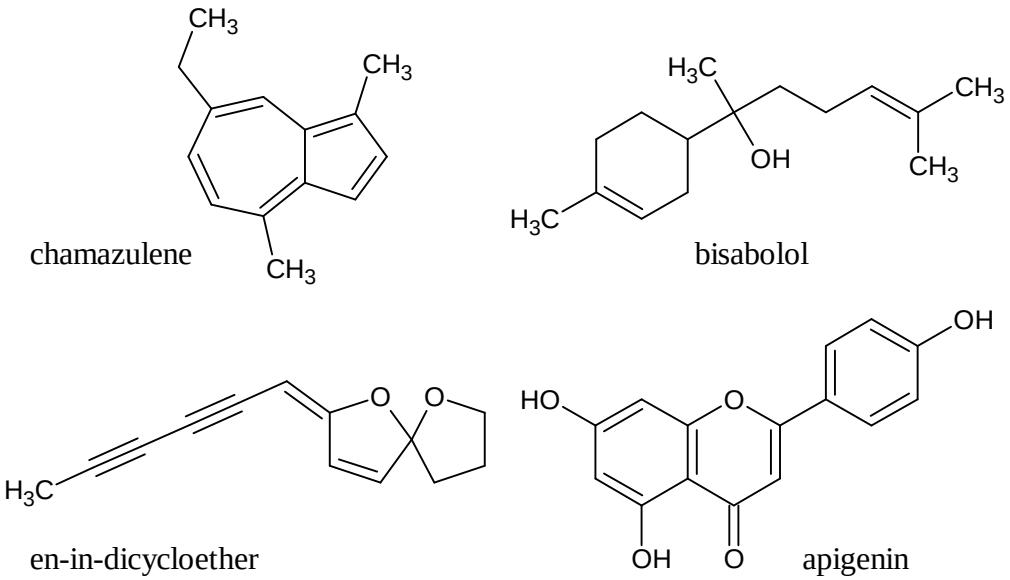


Figure 2. Chemical constituents of *Chamomillae flos*

In vitro, chamomile extracts inhibited both cyclooxygenase and lipoxygenase and thus the production of prostaglandins and leukotrienes, known inducers of inflammation [5]. Both bisabolol and bisabolol oxide have been shown to inhibit 5-lipoxygenase, but bisabolol was the more active. The antiinflammmatory effect of matricin, chamazulene, bisabolol and its oxides in various animal models, such as inhibition of carrageenin-induced rat paw oedema, have been demonstrated, although their activity was somewhat less than of salicylamide [6]. Topical application of either the total chamomile extract, or the flavonoid fraction, was very effective in reducing inflammation. Apigenin and luteolin were more active than indometacin and phenylbutazone. Intradermal application of liposomal apigenin-7-glucoside inhibited, in a dose dependent manner, skin inflammations induced in rats by xanthine oxidase and cumene hydroperoxide [7]. A hydroalcoholic extract of chamomile inhibited the growth of *Staphylococcus aureus*, *Streptococcus mutans*, group B *Streptococcus*, *Streptococcus salivarius* and it had a bactericidal effect *in vitro* on *Bacillus megatherium* and *Leptospira icterohaemorrhagiae*. *In vitro*, the volatile oil of chamomile also inhibited *Staphylococcus aureus* and *Bacillus subtilis* [8]. It acts as spasmolitic, antiphlogistic and helps healing wounds. Internal *Chamomillae flos* is used for symptomatic treatment of digestive ailments such as dyspepsia, epigastric bloating, impaired digestion and flatulence [9, 10]. Infusions of this drug have been used in the treatment of restlessness and in mild cases of insomnia due to nervous disorders [11]. External it is used against inflammation and irritations of the skin and

mucosa (skin cracks, bruises, frostbite and insect bites), including irritations and infections of the mouth and gums and haemorrhoids. Inhalation - symptomatic relief of irritations of the respiratory tract due to the common cold.

Preparations of this drug are: dried flower heads, liquid extract (1:1 in 45% alcohol), tinctures and other galenicals. If used internally adult, average daily dose of flower heads is 2-8 g usually divided in 3 doses a day. Fluid extract is usually used in ratio 1:1 to 45% ethanol with a daily dose of 1-4 ml, 3 times a day. Child dose of flower heads is up to 2 g, 3 times daily and of fluid extract half of the dose for adults. However, ethanol extracts should not be used by children under 3 years of age. Externally chamomile can be used for compresses, rinses or gargles as 3-10% (30-100 g/l) infusion, 1% fluid extract or 5% tincture. If used for baths concentrations are lower 5 g/l (0.5%) of water or 0.8 g/l (0.08%) of alcoholic extract. For vapour inhalation 6 g of the drug or 0.8 g of alcoholic extract are mixed per litre of hot water [1]. The presence of lactones in *Chamomillae flos* - based preparations may cause allergic reactions in sensitive individuals and there have been reports of contact dermatitis due to chamomile preparations [12]. It should be noted that very few cases of allergy specifically attributed to German chamomile [13]. A few cases of anaphylactic reactions to the ingestion of *Chamomillae flos* have also been reported. Chamomile is contraindicated in patients with a known sensitivity or allergy to plants of the Asteraceae such as ragweed, asters and chrysanthemums. No information available concerning general precautions, drug interactions, drug and laboratory test interactions, non-teratogenic effects on pregnancy, nursing mothers, or paediatric use.

Substitutes and adulterants identified for this plant are: *Anthemis nobilis* L., *Anthemis cotula* L., *Anthemis arvensis* L., *Matricaria inodora* L. (*Tripleurospermum inodorum* L.). *Chamomilla recutita* has a conical hollow receptacle, while the substitutes and adulterants have cylindrical or globular receptacle.

Chamomile is used in a variety of cosmetic products (Kamagel®, Decumil® cream), also as drops for relieving pains during the growth of teeth in babies (Miradent®) [14, 15]. It is one of the constituents in *Unguentum haemorrhoidale* [16]. Most of the commercially available products of chamomile are monocomponent and traditional, so the commercial production could be focused on combinations with other herbal drugs and new formulation like patches for dermatological purpose.

3. *Lavandula officinalis*

Lavandula officinalis (L.) Chaix ex Mill. (Fig. 3) is known under these names: lavender (Eng.), Lavendel (Ger.), lavande (Fr.). *Lavandulae flos* consists of the dried flowers of *Lavandula officinalis* (L.) Chaix ex Mill. (synonyms: *L. angustifolia* Mill. and *L. vera* DC.), Lamiaceae [1].

Lavandula officinalis grows as shrub up to 60 cm in height. The leaves are narrow lanceolate and the flowers are bluish tubular-bilabiate. Lavender prefers a temperate climate, so it is present in Mediterranean region. It is mainly cultivated in France, Croatia (islands), Spain and Bulgaria for the distillation of an essential oil. Flowers are used for medicinal purpose. The drug *Lavandulae flos* contains essential oil - *Lavandualae aetheroleum* which is produced by water distillation; pale yellow in color. To preserve the essential oil flowers should be collected before flowering [17].

Lavender contains an essential oil, tannins, flavonoids, phenolic acids, coumarins and phytosterols. *Lavandulae aetheroelum* contains linalyl acetate, linalool, borneol, isoborneol, cineole and camphor in smaller amounts. Structures of major constituents of an essential oil are shown on Fig. 4 [17].

Figure 3. *Lavandula officinalis*

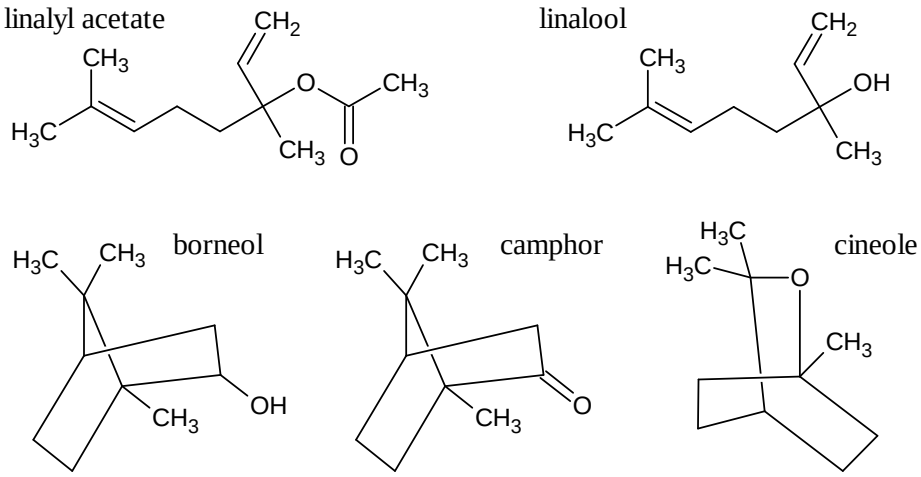


Figure 4. Major constituents of *Lavandulae aetheroleum*

Based on comparison of SCF-CO_2 and hydrodistillated essential oil linalyl acetate has been identified as the major antibacterial component. Antimicrobial effect of linalyl acetate may result, at least partially, from a perturbation of the lipid fraction of microorganism plasma membrane, resulting in alterations of membrane permeability and in leakage of intracellular materials. Penetration and interaction with intracellular site critical for antibacterial activity is also plausible site of action on the basis of lipophylicity [18]. Linalool is one of the components responsible for antifungal activity that was demonstrated on *Candida albicans* [19]. Linalyl acetate relaxes the vascular smooth muscle through partially activation of nitric oxide/cyclic guanosine monophosphate pathway and partially MLC dephosphorylation via activating MLC phosphatase [20]. Analgesic activity of lavender oil is achieved via opioidergic as well as cholinergic pathways, but no activity was detected with linalyl acetate and linolool on their own [21]. Antiinflammatory effect of linalool and linalyl acetate as its prodrug has been shown on rats.

Lavender is applied as a mild sedative, antidepressant, cholagogue, spasmolytic, carminative, stomachic and diuretic [22, 23]. Anticonflict effect of lavender oil was shown on mice [24]. The oil also acts as rubefacient and antirheumatoid, antibacterial and antifungicide [19, 25]. It has been shown that essential oil has supportive antiinflammatory effects in bronchitis. Applied topically it causes hyperemia, so it is often used in cosmetic products; the bath with essential oil calms and relaxes. Internally used as tea; mainly acts as carminative [26].

Dried flowers of lavender (1-2 g) are used in form of infuse for good-night sleep.

Tincture is prepared in ratio 1:6 with 60% ethanol and is used in dose of 2-4 ml [27].

Allergies (manifested as contact dermatitis) have been described for some components of essential oil e.g. linalyl acetate that oxidizes to epoxide and hydroperoxide [28]. High levels of essential oil can act as irritants. Precaution should be exercised when used by pregnant women and small children.

Lavandula latifolia and *L. hybrida* are used for production of essential oil in cosmetic industry. These oils contain significant amounts of camphor so they act as repellents.

Inhalator is used for clearance of block air ways (Sinusan®) [29]. It also comes in combination with ketoprofen to relief pains (Fastum Gel®, Ketogel®) [30]. *Lavandulae spiritus* is prepared as 2% *Lavandualae aetheroleum* in 90% ethanol [16].

Lavender is a thermophile plant that requires a lot of sunlight and high temperatures and as non-request plant grows also on stone and unfruitful soil. Those conditions are completely fulfilled in the whole Littoral Croatia. The centre of lavender production in Croatia is Hvar Island, which with 130 sunny days per annum is ideal for its cultivation. Lavender can also be cultivated in areas with lower average temperatures, but in such conditions the quality of its oil is significantly reduced. Lavender is cultivated on ca. 200 hectares in Croatia and it accounts for 11% of medicinal plants produced. Only about 60 to 80 tons of lavender are grown on Hvar Island every year.

4. *Vitex agnus-castus*

Vitex agnus-castus L. belongs to the family Verbenaceae and is known under the names: chaste tree (Eng.) and Moenchpfeffer, Keuschlam (Ger.) [31].

Chaste tree is a deciduous shrub, which reaches height of up to 5 m. The leaves are opposite, hand-shaped, composed of five to seven radiating leaflets which are borne on a main stalk. The leaflets are linear, lance-shaped, toothed, dark green above and grey beneath with a very close felt. From August to October flower panicles with numerous flowers are formed. The flowers are fragrant and of blue, lilac, rose or white color. The berries resemble peppercorns, hard, with a purple to black skin, yellowish within, half-covered by their sage-green calyces and containing four seeds. The plant is shown on Fig. 5 [32, 33].

Chaste tree is found growing wild in Mediterranean costal region and Central Asia. In Croatia, it grows along the costal sand shores of Adriatic Sea and river banks in Istria and

Dalmatia. Fruits are used for medicinal purpose. *Agni casti fructus* contains dried black fruits of chaste tree similar to pepper.

Figure 5. *Vitex agnus-castus*

Chaste tree contains essential oil (α-pinene, sabinene, β-phellandrene, 4-terpineol and sesquiterpenes), iridoids (agnuside, aucubin), flavonoids (casticin, isovitexin, isovitexin xylosid, orientin, isoorientin, penduletin and chrysosplenol D), diterpenes of the labdane and clerodane types (rotundifuran) and Δ4-3-ketosteroid hormones. Some constituents of *Agni casti fructus* are presented on Fig. 6 [33, 34].

In vitro and *in vivo* experiments have shown that chaste tree has dopaminergic, prolactin inhibiting activity. It binds to striatal D2 receptors, acting as prolactin-inhibiting factor on prolactin producing lactotropic cells of pituitary gland. This is mainly attributed to labdane diterpenoids. Estrogen activity was attributed to flavonoids, but other substances could also be responsible for the activity of extract (e.g. linolenic acid) [36].

This plant is used as a popular remedy for women cyclic disorders (polymenorrhea, oligomenorrhea, amenorrhea). Used internally it increases levels of estrogen and progesterone via hypophysis and also decreases levels of prolactin. It is used in gynecological disorders

like: menopausal symptoms, PMS (mastodynia, mastalgia), dysmenorrhea, female sex hormone imbalances or deficiencies. It is used in a form of tablets or drops. Daily amounts used should be equivalent of 30 to 40 mg of ethanolic tincture. To achieve results, it should be used for several months. Rare side effects are: rash, headache, hair loss, nausea itching and urticaria on skin. *Agni casti fructus* sorters or prorogate period between menstruations. This plant is not to be used in pregnancy [37-39].

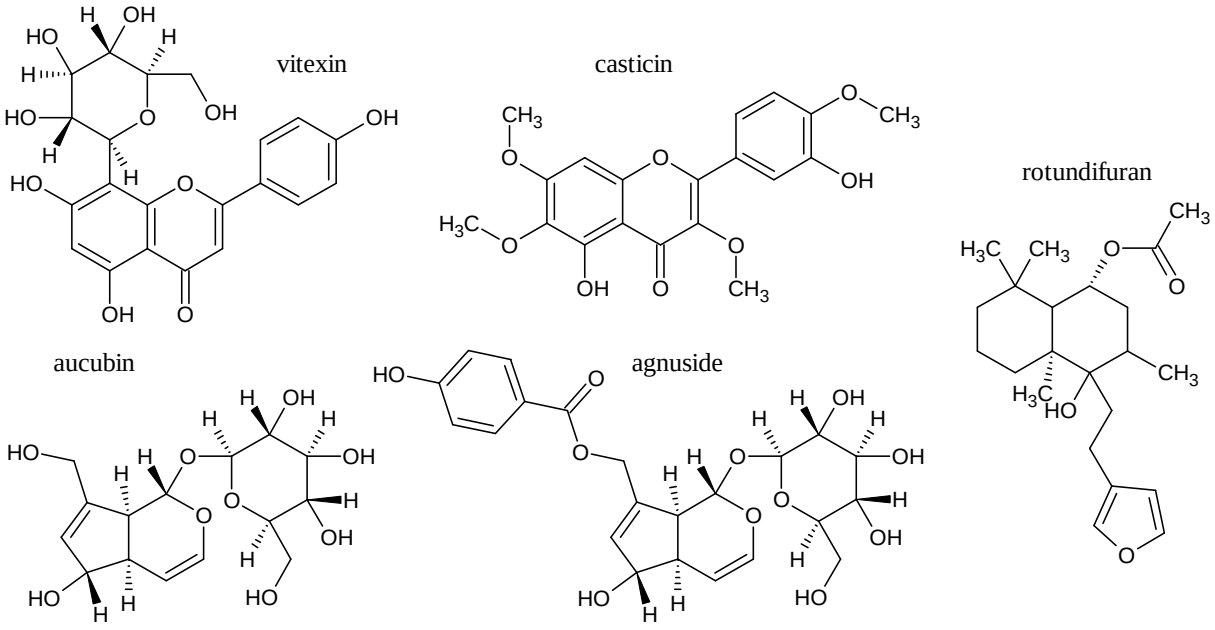


Figure 6. Some constituents of *Agni casti fructus*

After collecting the fruits, aquatic-ethanolic tincture (50-70%) is prepared. It can be used as tincture or dried for capsules. Agnolyt® (Germany) is monocomponent product that comes in the form of capsules and solution containing ethanol extracts of chaste tree [40]. Film tablets are known under trade name Agnucaston® [41]. Mastodynon® (Germany) drops are ethanolic solution containing active components of: *Vitex agnus-castus*, *Caulophyllum thalictroides* (L.) Michx., *Cyclamen* sp., *Ignatia* sp., *Iris* sp. and *Lilium triginum* L. [42]. It also comes under these trade names: Alyt®, Femicur® N, Prefemin®, PreMens®, Strotan® [38]. Most of the commercially available products come from Germany, so the potential of worldwide production is significant. As there is no standardized product on a basis of characteristic substances, this presents possibility for development.

5. *Sambucus nigra*

Sambucus nigra L. belongs to the family Caprifoliaceae (Sambucaceae) and is known under these names: Black elder, common elder bourtree (Eng.), schwarzer Holunder, Hollerbusch (Ger.), sureau noir (Fr.), sambuco nero (Ital.). It is a shrub or small tree up to 10 m high, with brownish-grey bark and white pith. Stems are often with strong, erect shoots from base - branches often arching. Leaves are 20 cm in length, with 5-7 leaflets. Leaflets are 3-9 cm long, ovate, pointy, serrate and rarely pubescent beneath. Stipules are absent or very small. Inflorescence flat topped, 10-20 cm in diameter, with 5 primary rays. Corolla is approximately 5 mm in diameter; flowers are creamish white and anthers cream. Fruit is a black drupe, globose, 6-8 mm, sometimes greenish-yellow, containing 3-5 seeds. The plant is shown on Fig. 7 [43, 44].

Figure 7. *Sambucus nigra*

Black elder is widespread in Europe, West and Central Asia and North Africa. Flowers and fruits are used for medicinal purpose.

The drug, *Sambuci flos*, contains flavonoids, phenolic acids (coumaric, caffeic, ferulic, chlorogenic), the essential oil, triterpenes (α- and β- amyrin, lupeol), triterpenic acids (ursolic, oleanolic), sterols (cycloartenol), flavonoids (astragalin, rutin, isoquercitrin = isoquercetin,

hyperoside, quercetin, kaempferol), cyanogenic glycosides (sambunigrin), free fatty acids (linolic, linolenic, palmitic) and tannins. Fruits contain a lot of tannins, cyanogenic glycosides, vitamins (C, B) and fruit acids. Some constituents of *Sambuci flos et fructus* are presented on Fig. 8 [45].

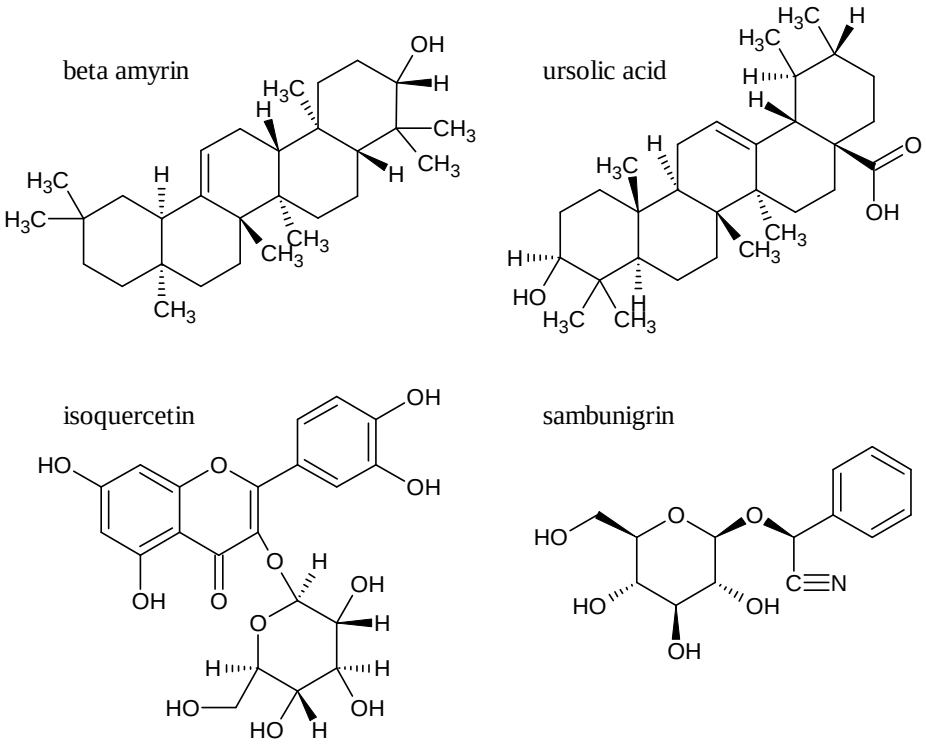


Figure 8. Major constituents of *Sambuci flos et fructus*

It is not known which constituent is responsible for increased production bronchial mucosa and sweatening. β-amyrin has autoprotective and ursolic acid antiinflammatory effect. Elderberry constituents neutralize the activity of the hemagglutinin spikes found on the surface of several viruses. It also significantly increases cytokine production (tumor necrosis factor-alpha and interleukins 1β, 6 and 8). It has also been shown that anthocyanins have high antioxidant activity.

Black elder acts as diaphoretic, bronchial secretolytic, antiinflammatory and antiviral (Herpes virus, influenza A and B) agent, hepatoprotective, anticatarrhic and antidiabetic. *Sambuci flos* is used as diaphoretic and laxative. It is used in common colds. In folk medicine, the drug is also used in preparations of gargles. Average daily dosage is 10-15 g of drug. It is

used as infusion 3-4 g per cup (150 ml) as hot as possible. Liquid extract is prepared in ratio 1:1 with 25% ethanol and is used in doses 2-4 ml [46-48].

Substitutes of this plant are: *Sambucus canadiensis* L.; adulterants: *Sambucus ebulus* L., *Sambucus racemosa* L. and *Filipendula ulmaria* (L.) Maxim [49].

Black elder is used in case of common cold, flue and sinusitis as tea or water tincture. It can be used in combination with other herbal drugs e.g. galenic formula *Species laxantes* that contains *Sennae folium* 20, *Sambuci flos* 20, *Frangulae cortex* 35, *Foeniculi fructus* 15, *Kalii natrii tartaras* 10 and *Aqua purificata* 10 [16, 50]. Sambucol® Black Elderberry Extract also comes as lozenges for sore throats [51].

6. *Hypericum perforatum*

Hypericum perforatum L. (family Hypericaceae) is found under these names: St. John's Wort (Eng.), Johanniskraut (Ger.), le mille-pertuis (Fr.) and isperico pilatro (Ital.). This plant is herbaceous, aromatic rhizomatous perennial up to 1 m high, glabrous throughout, with simple, opposite, sessile, ovate to linear leaves sprinkled with oil translucent glands. Flowers are bright yellow and arranged in a broadly paniculate, compound cymose inflorescence at the top of steam. Five petals are asymmetric, with hypericin containing black nodules. The plant is shown on Fig. 9 [52, 53].

Figure 9. *Hypericum perforatum*

Hypericum perforatum is widely distributed in Europe, western Asia and throughout the United States. The drug, *Hyperici herba*, consists of the aerial parts: leaves and flower tops.

Hypericin and hypericin-like substances (derivates of naftodianthrone) are main active components of herb. This drug contains flavonoids (rutin, quercitrin, isoquercitrin, hyperoside, phenolic acids (chlorogenic, caffeic), phloroglucinol derivates (hyperforin and adhyperforin), essential oil (methylbutenol), small amounts of procyanidins and tannins (up to 15% of catechins). Biflavonoids, biapigenin and amentoflavone, are characteristic constituents - markers of flowers. Major constituents of *Hypericum perforatum* are presented on Fig. 10 [54-56].

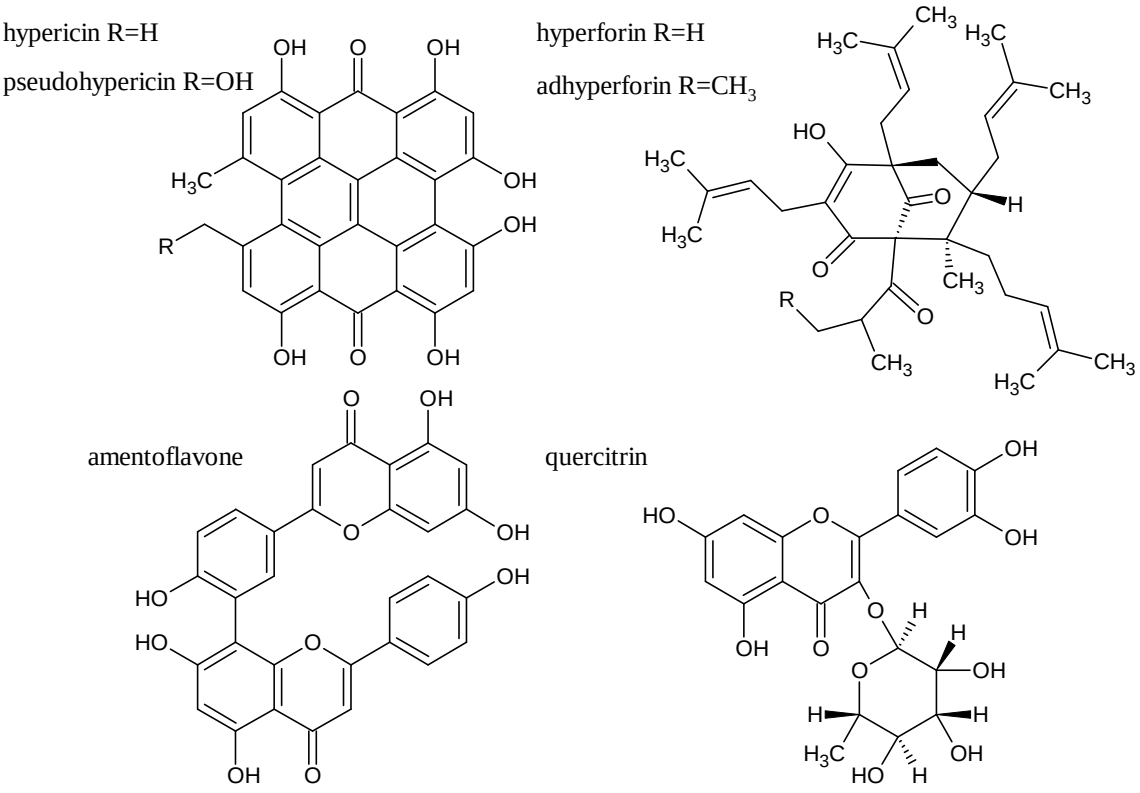


Figure 10. Major constituents of *Hyperici herba*

The drug includes more components of which naphthodianthrones and phloroglucinols are the most important. Hypericin increases light utilization during winter period and flavonols decrees disintegration of neurotransmitters (via catechol-O-methyl-transferase). It has been shown that hydroethanolic extract has high affinity of binding to GABA-A, GABA-

B, adenosine, benzodiazepine, inositol triphosphate, MAO-A and MAO-B receptors. It also modulates serotonin and dopamine neurotransmission. Tannins produce constriction due to interactions with proteins and thus it is used as astringent [57].

The drug is used as sedative, astringent, antiseptic, analgesic, relaxant. St. John's Wort extract shows antimicrobial activity especially against *Candida albicans*. The herb is used internally for the treatment of psychogenic disturbances, depressive states and/or nervous excitements, as an antiphlogistic agent in the treatment of inflammation of the bronchi and urogenital tract, treatment of biliary disorders, bladder irritation, common cold, diabetes mellitus, dyspepsia, as a diuretic, emmenagogue and antimalarial agent. Externally as oily preparations for the treatment and after-treatment of incised and contused wounds, skin ulcers and first-degree burns. It is recommended in case of "burned out syndrome" (improves concentration, memory and receptivity) and muscle pain (myalgia) [58, 59].

Average daily dosage for internal use is 2-4 g of drug or equivalent of 0.2-1 mg of total hypericin. It is used as tea, tincture (20-30 drops) or solid preparation for at least six weeks. Oil is used internally for dyspepsia and externally for wounds, burns and frost bites.

Some of the side effects include: allergic skin reaction, increased prothrombin time, gastrointestinal complaints. Hypericin may cause photosensibilisation - rare, usually appears in fair-skinned individuals. *Hyperici herba* should not be used with other antidepressants without professional guidance. Interactions are possible with oral contraceptives, anticoagulants and theophylline on cytochrome enzyme CYP3A4. Precaution should be practiced when used with antiretrovirotics, immunosuppressants, anticonvulsives and digoxin [27].

Only the flowering tops of the plant are collected from mid-June to August, as leaves and flowers have the most active components. The extract is standardized to 0.3% of hypericin and 2-5% of hyperforin.

Substitutes and adulterants of this plant are: *Hypericum barbatum* Jacq., *H. maculatum* Crantz., *H. hirsutum* L., *H. montanum* L., *H. tetrapterum* Fr.

Infuse is prepared as monodrug or in combinations with e.g. rosemary, primula. Tincture is made in ratio 1:5 with 70% ethanol. Oil is made of fresh flowers in ratio of 1:10 with olive oil and is left for three weeks in the sun. Some of the commercially available products are: Aktivin H®, St. John's wort balm, St. John's wort oil, Hyperiforce®, Jarsin® 300, Kira®, Remotiv® [16].

Hypericum is grown as a commercial crop in parts of Europe and North America, all the while being considered a noxious weed in more than 20 countries. With the recent

resurgence in its medicinal use there is a significant market for this plant. Demand is considerably higher for certified organic product. Growers are advised to contact companies involved in the medicinal herb industry to determine markets before starting large-scale cultivation. Small growers may not be able to meet the demand of large quantity buyers. Saint-John's wort known as Johanniskrant in German for centuries has been used to treat people with mild and moderate depression without the side effects of Prozac. Widely sold in Germany and other European countries, awaiting official approval by the US Food and Drug Administration, Saint-John's wort is being regarded as a serious rival to Prozac [60-62].

Hypericum is used worldwide and was the fifth bestselling dietary supplement in USA. The plant is sold as a dry bulk herb, or as capsules, tinctures and tablets. *Hypericum* was in high market demand in North America in the late 1990's. This demand dropped noticeably after 2000, but is now picking up again. In 2004 *Hypericum* ranked 9th in dollar sales among herbal dietary supplements in food, drug and mass market retail channels in the USA. Statistics are not available for Croatian production and sales.

7. *Rhamnus frangula*

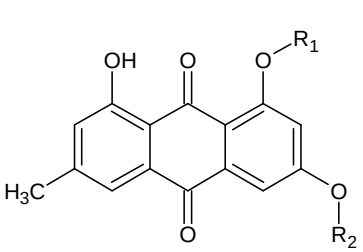
Rhamnus frangula L. (synonym: *Frangula alnus* Mill.) belongs to the family Rhamnaceae. This plant is found under these names: Alder buckthorn, berry-bearing alder (Eng.), Faulbaum (Ger.), frangule, nerprun bourdaine (Fr.), frangula, fragola (Ital.). It is a bush that grows up to 3 m, with opposite, finely serrated leaves, on branches often terminating in spines. Small, yellowish-green, axillary flowers are arranged in cymes. Fruits are shiny and black when ripped. The bark has whitish, horizontal lenticels. The plant is shown on Fig. 11 [1, 63].

Buckthorn is distributed across Europe, North Africa and Asia.

The bark is used for medicinal purpose. The drug, *Frangulae cortex* (synonym *Rhamni frangulae cortex*) is used in constipation. In folk medicine, the drug is also used as diuretic.

The drug contains a number of constituents, including anthracene derivatives, tannins, flavonoids, phenolic acids, pectins, saccharides, ascorbic acid and small amounts of peptide alkaloids (frangulanin, franganin). Anthraquinones are responsible for laxative effect and can be divided in two types: emodin-physcion and chrysophanol. They come as glycosides (e.g. glucofrangulins A and B, frangulins A, B and C) and in small amounts as free anthraquinones and dianthrones. Major constituents of *Frangulae cortex* are presented on Fig. 12 [64, 65].

Figure 11. *Rhamnus frangula*



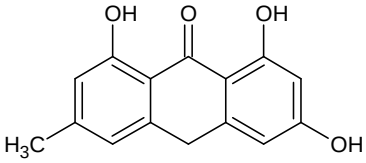
emodin (R_1=H, R_2=H)

frangulin A (R_1=H, R_2=Rha)

glucofrangulin A (R_1=Glc, R_2=Rha)

frangulin B (R_1=H, R_2=Api)

glucofrangulin B (R_1=Glc, R_2=Api)



emodin anthron

Figure 12. Major constituents of *Frangulae cortex*

Antraquinone glycosides are hydrolyzed in colon to anthrones. Anthrons (1,8-dihydroxy-anthracene derivates) increase motility of colon by inhibiting stationary and stimulating propulsive contractions. The result is accelerated intestine passage and reduction in liquid absorption through lumen (shortened contraction time). Anthraquinons stimulate mucous and active chloride secretion resulting in enhanced fluid secretion [66].

Frangulae cortex acts as a laxative, 8 to 12 hours after administration. This drug is used at constipation and other conditions in which soft stool is desirable (hemorrhoids, anal

fissures, post rectal-anal operations). The drug can be used as tea (2 g per cup), but it is recommended to be used in standardized products. Average dose of dried extract is 0.5 g. Standardized drugs to hydroxyanthracene derivates should be used in dose 20-30 g of glucofrangulin A equivalent. Dried extract can be used for making decoct, cold maceration or elixir. Individual dose should be adjusted - minimum amount of drug for the effect, in total less than daily dose [66].

Red urine is possible due to antraquinone content. In chronic constipation (elderly) disbalance of electrolytes, primarily potassium, is possible; cramps in stomach and diarrhea; emesis if used fresh.

It is prohibited to use this drug at intestine obstructions, acute intestinal inflammation (morbus Crohn, colitis ulcerosa), appendicitis, abdominal pain of unknown origin, pregnancy, lactation, children age 12 or under. It should not be used for a longer period, as the effect fades and dose has to be increased (intestinal sluggishness). Interactions are possible on level of absorption due to shortened period of retention in intestinal. As the level of potassium is lowered interactions have been noticed in combinations with cardiac glycosides, antiarrhythmics, thiazide diuretics, corticosteroids [27].

The bark of tree is collected from May to July and dried for one year or by heating to 100 °C for 1 to 2 hours thus disintegrating reduced anthrone and dianthrone glycoside forms to the oxidized forms (glucofrangulins are partly decomposed to the frangulins or frangula-emodin-8-O-glucoside and to the aglycone frangula-emodin). Dry extract of *Frangula* is prepared by percolation in ratio 2:1 with 70% ethanol that is evaporated under low pressure and dried in vacuum [27].

Possible substitute is *Rhamnus catharticus*, but in this case fruits are used as a drug (*Rhamni cathartici fructus*). It contains emodine, glucofrangulin and frangulin. The adulterates are barks from *Rhamnus fallax* Boiss., *Rhamnus purshianus* DC., *Prunus padus* L. and *Alnus glutinosa* Gaertn.

In Croatia following formulations are available: Purgal® tablets, Alofran® tablets, Planinski čaj® and galenic tea *Species laxantes* [16, 30].

8. *Rosmarinus officinalis*

Rosmarinus officinalis L., (Eng. rosemary, Ger. Rosmarin) is an evergreen, aromatic shrub belonging to the family Lamiaceae. It attains a height of 1-2 m and has slender, ash-

colored branches. The leaves of this plant are approximately 3.5 cm long, 2 to 4 mm broad, rigid, opposite, sessile and linear. The upper surface is dark green and glossy, whereas the lower surface is gray and woolly owing to the presence of numerous branched hairs. Rosemary bears verticillacters of mauve flowers which have campanulated, two-lipped calyx and a widely-gaping, two-lipped corolla. It has characteristic aromatic odour and aromatic, pungent and slightly bitter taste. The plant is shown on Fig. 13 [1, 67].

Figure 13. *Rosmarinus officinalis*

Rosemary is widely distributed in France, Croatia (Dalmatian islands), Spain and North Africa. Leaves (*Rosmarini folium*), as well as essential oil (*Rosmarini aetheroelum*) are used as herbal drugs.

The leaves of rosemary contain an essential oil (1.5-2.5%), flavonoids (apigenin, luteolin, nepetin), triterpenic acids (ursolic and oleanolic), diterpenes (carnosol, rosmaridiphenol, rosmanol) and significant amounts of tannins, mainly as rosmarinic acid. The main components of essential oil are: 1,8-cineole (30%), borneol, bornyl acetate,

camphor, camphene, α-pinene and α-terpineol. Major constituents of *Rosmarini aetheroleum* are presented on Fig. 14 [68].

Flavons and rosmarinic acid act as antioxidants thus making the rosemary natural industrial conservant [69]. Rosmarinic acid acts as antiphlogistic and prevents inflammation. Essential oil is used for pains in joints and muscles, stimulating nervous and vascular systems. Experimental data shows that the drug acts as antispasmodic on gall passages and small intestines, has positive inotropic effect and increases flow through the coronary artery.

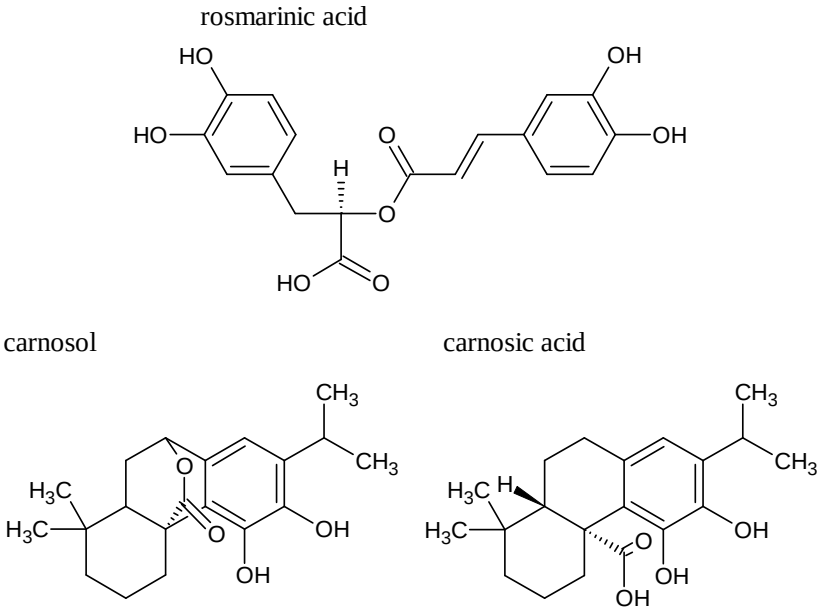


Figure 14. Major constituents of *Rosmarini aetheroelum*

It has been suggested that carnosol could be used in treatment of Parkinson's disease as it protects dopaminergic neuronal cells through downregulation of capase-3 [70]. Carnosol and carnosic acid activate human peroxisome proliferator-activated receptor gamma (nuclear hormone receptor) and thus have potential to lower glucose and fatty acids blood levels; this can also explain their antiinflammatory and antiproliferative effects [71].

Rosemary acts as carminative, stomachic, spasmolytic, thymoleptic, sedative, emmenagogue, diuretic and also shows antibacterial and antifungal activity. Used topically it acts as rubefacient and is used as supportive therapy of rheumatic diseases and circulation problems. It promotes wound healing and is used as mild antiseptic. Internally is used in dyspepsia, flatulence, as well for improvement of hepatic and biliary function. It also helps

with headaches, migraines as well caused by hypertension [72, 73].

Dried herb can be used as infuse in doses of 2 to 4 g. It should be used twice a day with a meal, not in the evening as it acts as stimulant. Extract is produced in ratio 1:1 with 45% ethanol and is used in doses of 2-4 ml.

Rosemary leaves and its preparations, especially those containing carnosol can cause contact dermatitis and hypersensitivity.

Baths should be avoided by patients with large, open wounds, large skin lesions, feverish conditions or acute inflammation, severe circulatory disorders or hypertension. It is not recommended to be used in pregnancy [27].

Rosmarini folium contains fresh or dried leaves, gathered while flowering and *Rosmarini aetheroleum* is obtained by water distillation.

Very similar, but poisonous plant is *Andromeda polifolia* L.

The drug is used as tea, tincture or essential oil. Rosemary can also be used in a form of medicinal wine and is prepared by maceration of 20 g of drug in 1 litre of wine for 5 days. In Croatia, it is mainly present in creams, balsams and gels like Reumasan® and Veneforte® that are used for better peripheral circulation [29].

Most of the commercially available pharmaceutical products are polycomponent. In Croatia rosemary is mainly used as spice. But, rosemary has many uses besides culinary. It is used as a medicinal, an aromatic, an ornamental in the landscape, as a dye, in cosmetics and as a houseplant. Rosemary essential oil adds a piney scent to soaps, creams, lotions, perfumes and toilet water.

9. *Salvia officinalis*

Salvia officinalis L. is found under these names: Shop-sage (Eng.), Edler Salbei (Ger.), sauge, serve (Fr.), salvia, erba savia (Ital.). Sage is a ca. 70 cm tall subshrub belonging to the Lamiaceae family. The leaves of sage are long-petiolate, 3 to 10 cm long and up to 3 cm wide, oval, oblong-ovate to lanceolate, olive-grey and densely pubescent on both surfaces. The flowers are ca. 2 cm long, mostly with a bluish violet corolla, arranged in whorls forming a loose spike. The dry plant is shown on Fig. 15 [1, 74].

Sage is Mediterranean as well as continental plant, but the original effects the composition and quality of the essential oil.

For medicinal purpose leaves are used - *Salviae folium*. The drug *Salviae folium* contains the essential oil - *Salviae aetheroleum* which is produced by water distillation, nearly colorless.

Figure 15. *Salviae folium*

The drug (*Salviae folium*) contains the essential oil (1-2.5%), tannins (3-7% of Lamiaceae type), flavonoids (salvigenin), phenolic acids, diterpenoids (carnosol and carnosic acid) and triterpenes (ursolic and oleanolic acids, germanicol). Essential oil, *Salviae aetheroleum*, contains thujone (α and β, 60%), 1,8-cineole, camphor, borneol, bornyl acetate. Major constituents of *Salvia officinalis* are presented on Fig. 16 [75].

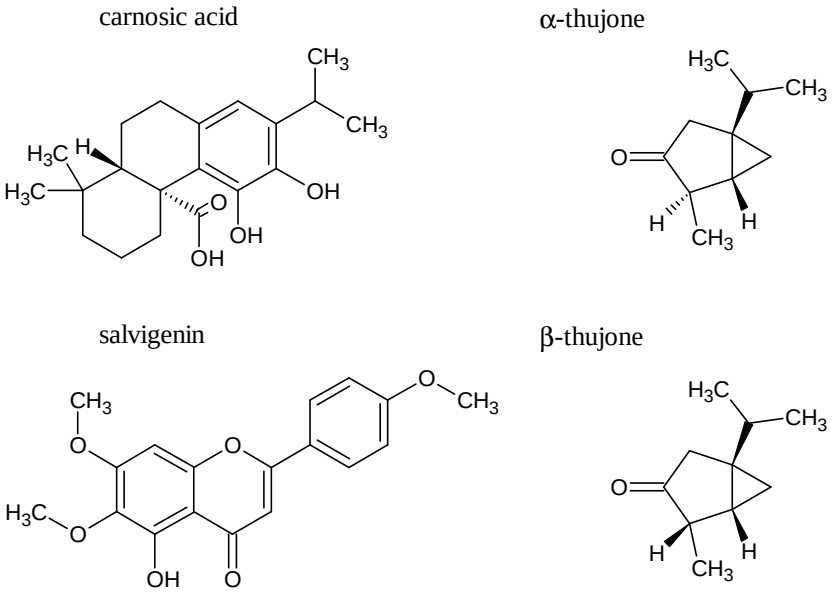


Figure 16. Major constituents of *Salvia officinalis*

Rosmarinic acid and triterpenic acids have been shown to have antiinflammatory properties. By inhibiting cholinesterase dried extract of sage improved mood and cognitive performance following the administration of single doses to healthy young participants. This mechanism could also explain beneficial effects in supportive therapy of Alzheimer disease. Carnosic acid inhibits pancreatic lipase and significantly inhibit triglyceride elevation. It has been shown that polysaccharide fraction of sage has antiinflammatory effect [27, 76-78].

Used internally, sage acts as antiseptic, bacteriostatic, fungistatic and virustatic. It prevents inflammation and is used as garganta. Due to high content of Lamiaceae tannins it acts as adstringent, antidiaroic, as well as stomachic and carminative [79, 80]. Sage is used as antiphlogistic for the inflammation of mouth and throat, for gingivitis and stomatitis, mainly in the form of the gargle but also as tea for digestive complaints, flatulence, inflammation of the intestinal mucosal, in diarrhea and as antihydrotic. It is also used to lower the excessive perspiration (hyperhidrosis) and inhibit secretion [81].

Dried herb can be used as infuse in doses of 1 to 4 g. Extract is produced in ratio 1:1 with 45% ethanol and is used in doses of 1-4 ml. Daily dose should be limited to 4-6 g of herb, 0.1-0.3 g of essential oil, 2.5-7.5 g of tincture or 1.5-3 g of fluidextract.

If used in amounts of more than 15 g of leaves or 2 g of essential oil it can cause dryness of mouth, increased perspiration and pulse and dizziness. After prorogated ingestion epileptiform convulsions can occur. Sage should not be used by children and pregnents. In hyperhidrosis treatment for 2-4 weeks is recommended. Internal use of sage ethanol extracts should be limited to three days, as it contains toxic thujone.

The leaves are collected before flowering and dried. Essential oil is obtained by water distillation of young green parts of herb and leaves. Pulvis is used as aromatic spice. Substitutes and adulterants: occasionally, with the leaves of other *Salvia* species, principally those of *Salvia triloba* L., Greek sage; these have a white, velvety tomentum on both surfaces, which is denser than that of *S. officinalis*. The trichomes on the upper surface are not tortuous and whip-like, but are straight and stiff. TLC examination set out above, adulteration can be recognized by the divergent composition (a higher cineole and lower thujone content). Differentiation is also possible on the basis of the flavonoid profile.

In Croatia sage is used as antiseptic in the form of pastilles (Salviol®) and intimate care soap (Hygieia®) [82]. From folk medicine the use of sage leaves prepared in hot milk, sweetened with honey is recommended [27, 83].

As natural antiseptic, it has significant potency to be used in inflammations of upper respiratory track having in mind that in 80% of cases these inflammations are caused by

viruses and thus antibiotics should not be used. Alternative approaches to pharmaceutical forms like honey should be taken in consideration as sage is readily collected by bees.

ACKNOWLEDGEMENTS

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REFERENCES

1. British herbal pharmacopoeia. London: British Herbal Medicine Association, 1990.
2. Medić-Šarić M, Stanić G, Bošnjak I. The use of information theory and numerical taxonomy methods for evaluating the quality of thin-layer chromatographic separations of flavonoid constituents of *Matricariae flos*. Pharmazie. 2001; 56: 156-159.
3. Doelle B, Carle R, Mueller W. Flavonoidbestimmung in Kamillenextrakt-praeparaten. Dtsch Apoth Ztg. 1985; 125: 14-19.
4. Medić-Šarić M, Stanić G, Maleš Ž, Šarić S. Application of numerical methods to thin-layer chromatographic investigation of the main components of chamomile (*Chamomilla recutita* (L.) Rauschert) essential oil. J Chromatogr A. 1997; 776: 355-360.
5. Wagner H, Wierer M, Bauer R. *In vitro* inhibition of prostaglandin biosynthesis by essential oils and phenolic compounds. Planta Med. 1986; 52: 184-187.
6. Della Loggia R. Lokale antiflogistische Wirkung der Kamillen-Flavone. Dtsch Apoth Ztg. 1985; 125: 9-11.
7. Fuchs J, Milbradt R. Skin anti-inflammmatory activity of apigenin-7-glucoside in rats. Arzneim-Forsch. 1993; 43: 370-372.
8. Aggag ME, Yousef RT. Study of antimicrobial activity of chamomile oil. Planta Med. 1972; 22: 140-144.
9. Carle R, Isaac O. Die Kamille-Wirkung und Wirsamkeit. Z Phytotherap. 1987; 8: 67-77.
10. Casterline CL. Allergy to chamomile tea. J Am Med Ass. 1980; 244: 330-331.
11. Hormann HP, Korting HC. Evidence for efficacy and safety of topical herbal drugs in dermatology. Part 1. Anti-inflammatory agents. Phytomed. 1994; 1: 161-171.
12. Paulsen E, Anderson KE, Hausen BM. Compositae dermatitis in a Danish dermatology department in one year. Contact Dermatitis. 1993; 29: 6-10.

13. Hausen BM, Busker E, Carle R. Ueber das Sensibilisierungsvermoegen von Compositenarten. VII. Experimentelle Untersuchungem mit Auszuegen und Inhaltsstoffen von *Chamomilla recutita* (L.) Rauschert und *Anthemis cotula* L. Planta Med. 1984; 50: 229-234.
14. Kamagel: Patient information leaflet. [cited 2017 Jun 8]. Available http://www.krka-farma.hr/media/products/hr/otc/gen_pdf/2012/Kamagel.pdf.
15. Miradent. [cited 2017 Jun 8]. Available: http://www.miradent.de
16. Bećirević Laćan M, Begović-Dolinić V, Buhač I, Colnago F, Jurišić B, Medić-Šarić M, Nevečerel M, Smolčić-Bubalo A, Šušteršić T, Vrsalović M. Formulae Magistrales Croaticae. Zagreb: Hrvatska ljekarnička komora, 2010.
17. Maleš Ž, Medić-Šarić M. Selection of an optimal set of solvents in thin-layer chromatography of flavonoids and phenolic acids of *Lavandulae flos*. Pharmazie. 1999; 54: 362-364.
18. Peana AT, D'Aquila PS, Panin F, Serra G, Pippia P, Moretti MDL. Anti-inflammatory activity of linalool and linalyl acetate constituents of essential oils. Phytomed. 2002; 9: 721-726.
19. D'Auria FD, Tecca M, Strippoli V, Salvatore G, Battinelli L, Mazzanti G. Antifungal activity of *Lavandula angustifolia* essential oil against *Candida albicans* yeast and mycelial form. Med Mycol. 2005; 43: 391-396.
20. Koto R, Imamura M, Watanabe C, Obayashi S, Shiraishi M, Sasaki Y, Azuma H. Linalyl acetate as a major ingredient of lavender essential oil relaxes the rabbit vascular smooth muscle through dephosphorylation of myosin light chain. J Cardiovasc Pharmacol. 2006; 48: 850-856.
21. Ghelardini C, Galeotti N, Salvatore G, Mazzanti G. Local anaesthetic activity of the essential oil of *Lavandula angustifolia*. Planta Med. 1999; 65: 700-703.
22. Barocelli E, Calcina F, Chiavarini M, Impicciatore M, Bruni R, Bianchi A, Ballabeni V. Antinociceptive and gastroprotective effects of inhaled and orally administered *Lavandula hybrida* Reverchon "Grosso" essential oil. Life Sci. 2004; 76: 213-223.
23. de Moura Linck V, da Silva AL, Figueiró M, Piato ÂL, Herrmann AP, Dupont Birck F, Bastos Caramão E, Nunes DS, Moreno PRH, Elisabetsky E. Inhaled linalool-induced sedation in mice. Phytomed. 2009; 16: 303-307.
24. Umezu T, Nagano K, Ito H, Kosakai K, Sakaniwa M, Morita M. Anticonflict effects of lavender oil and identification of its active constituents. Pharmacol Biochem Behav. 2006; 85: 713-721.

25. Trombetta D, Castelli F, Sarpietro MG, Venuti V, Cristani M, Daniele C, Saija A, Mazzanti G, Bisignano G. Mechanisms of antibacterial action of three monoterpenes. Antimicrob Agents Chemother. 2005; 49: 2474-2478.
26. Hadžović S, Pilipović S. Ljekovito bilje i izrada preparata od ljekovitog bilja. Sarajevo: Šahinpašić, 1999.
27. Toplak Galle K. Hrvatsko ljekovito bilje. Zagreb: Mozaik knjiga; 2001.
28. Sköld M, Hagvall L, Karlberg A-T. Autoxidation of linalyl acetate, the main component of lavender oil, creates potent contact allergens. Contact Dermatitis. 2008; 58: 9-14.
29. Dietpharm. [cited 2017 Jun 8]. Available: http://www.dietpharm.hr
30. Croatian Agency for Medicinal Products and Medical Devices. [cited 2017 Jun 8]. Available: http://www.halmed.hr
31. E/S/C/O/P Monographs. Stuttgart: Thieme; 2003, pp. 8-13.
32. Antolić A, Maleš Ž. Quantitative analysis of the polyphenols and tannins of *Vitex agnus-castus* L. Acta Pharm. 1997; 47: 207-211.
33. Maleš Ž, Medić-Šarić M, Prstojević J, Puzović M. Application of numerical methods to TLC investigation of the essential fruit oil of *Vitex agnus-castus* L. Pharm Pharmacol Lett. 1997; 7: 50-52.
34. Hoberg E, Meier B, Sticher O. Quantitative high performance liquid chromatographic analysis of diterpenoids in *Agni-casti fructus*. Planta Med. 2000; 66: 352-355.
35. Maleš Ž. Determination of the content of the polyphenols of *Vitex agnus-castus* L f. rosea. Acta Pharm. 1998; 48: 215-218.
36. Jarry H, Spengler B, Porzel A, Schmidt J, Wuttke W, Christoffel V. Evidence for estrogen receptor beta-selective activity of *Vitex agnus-castus* and isolated flavones. Planta Med. 2003; 69: 945-947.
37. Blumenthal M. Herbal Medicine. Newton: Integrative Medicine Communications; 2000, pp. 62-64.
38. Blumenthal M. The ABC Clinical Guide to Herbs. Austin: American Botanical Council; 2000. pp. 61-72.
39. Daniele C, Thompson Coon J, Pittler MH, Ernst E. *Vitex agnus castus*: a systematic review of adverse events. Drug Saf. 2005; 28: 319-332.
40. Agnolyt: Patient Information Leaflet. [cited 2017 Jun 8]. Available: http://www.medapharma.de/fileadmin/user_upload/DE/Gebrauchsinformationen/Agnolyt_Madaus_GI_04_2015_2.pdf

41. State Agency of Medicines of the Republic of Latvia. [cited 2017 Jun 8]. Available: https://www.zva.gov.lv
42. Bionorica. [cited 2017 Jun 8]. Available: http://www.bionorica.de
43. Monograph: Sambucus nigra (elderberry). Altern Med Rev. 2005; 10: 51-54.
44. Plant for a Future Database. [cited 2017 Mar 2]. Available: http://www.pfaf.org/database/index.php
45. Maleš Ž, Medić-Šarić M. Investigation of the flavonoids and phenolic acids of *Sambuci flos* by thin-layer chromatography. J Planar Chromatogr. 1999; 12: 345-349.
46. Blumenthal M. The Complete German Commission E Monographs. Austin: American Botanical Council; 1998, p. 124.
47. Blumenthal M. Herbal Medicine. Newton: Integrative Medicine Communications; 2000. pp. 103-105.
48. Roxas M, Jurenka J. Colds and influenza: a review of diagnosis and conventional, botanical and nutritional considerations. Altern Med Rev. 2007; 12: 25-48.
49. Kabuce N. Invasive alien species fact sheet - *Sambucus nigra*. From: Online Database of the North European and Baltic Network on Invasive Alien Species - NOBANIS. [cited 2017 Mar 2]. Available: www.nobanis.org
50. Senjković R, Petričić V, Bećirević M. Drug formulations. Zagreb: Faculty of Pharmacy and Biochemistry; 1996, p. 5.
51. Sambucol. [cited 2017 Jun 8]. Available: http://www.sambucol.com
52. E/S/C/O/P Monographs. Stuttgart: Thieme; 2003, pp. 8-13.
53. Monograph: *Hypericum perforatum.* Altern Med Rev. 2004; 9: 318-325.
54. WHO monographs on selected medicinal plants. Geneva: World Health Organization; 2004, pp. 149-171.
55. Maleš Ž, Plazibat M, Bilušić-Vundać V, Žuntar I, Hazler Pilepić K. Thin-layer chromatographic analysis of flavonoids, phenolic acids and amino acids in some Croatian *Hypericum* taxa. J Planar Chromatogr. 2004; 17: 280-285.
56. Medić-Šarić M, Brantner A, Maleš Ž. Application of information theory and numerical taxonomy methods to thin-layer chromatographic investigations of *Hypericum perforatum* L. Acta Pharm. 1996; 46: 115-124.
57. Blumenthal, M. The ABC Clinical Guide to Herbs. Austin: American Botanical Council; 2000, pp. 321-334.
58. Blumenthal, M. The Complete German Commission E Monographs. Austin: American Botanical Council; 1998, p. 214.

59. Maleš Ž, Brantner AH, Sović K, Hazler Pilepić K, Plazibat M. Comparative phytochemical and antimicrobial investigations of *Hypericum perforatum* L. subsp. *perforatum* and *H. perfortum* subsp. *angustifolium* (DC.) Gaudin. Acta Pharm. 2006; 56: 359-367.
60. St. John's Wort, [cited 2017 Mar 2]. Available: http://www.naturenorth.com/summer/St_Johns_Wort/St_Johns_Wort2.html.
61. Andrews EL, Humble A. Herb as Rival to Prozac. Paris: International Herald Tribune; 1997.
62. Büter B, Orlacchio C, Soldati A, Berger K. Significance of genetic and environmental aspects in the field cultivation of *Hypericum perforatum*. Planta Med. 1998; 64: 431-437.
63. Blumenthal M. The Complete German Commission E Monographs. Austin: American Botanical Council; 1998, pp. 95-97.
64. WHO monographs on selected medicinal plants. Geneva: World Health Organization; 2003, pp. 114-123.
65. Medić-Šarić, M, Maleš, Ž, Šarić, S, Debeljak, Ž. Use of information theory and numerical taxonomy methods for evaluating the quality of thin-layer chromatographic separations of flavonoids and phenolic acids of *Rhamni cathartici* fructus. J Liq Chrom Rel Technol. 1999; 22: 83-103.
66. Blumenthal M, Goldberg A, Brinckmann J. Herbal Medicine. Newton: Integrative Medicine Communications; 2000, pp. 36-39.
67. E/S/C/O/P Monographs. Stuttgart: Thieme; 2003, pp. 429-436.
68. Medić-Šarić M, Maleš Ž. Application of numerical methods to the TLC analysis of flavonoids and phenolic acids in *Rosmarini folium*. J Planar Chromatogr. 1997; 10: 182-186.
69. D'Evoli L, Huikko L, Lampi AM, Lucarini M, Lombardi-Boccia G, Nicoli S, Piironen, V. Influence of rosemary (*Rosmarinus officinalis*, L.) on plant sterol oxidation in extra virgin olive oil. Mol Nutr Food Res. 2006; 50: 818-823.
70. Kim SJ, Kim JS, Cho HS, Lee HJ, Kim SY, Kim S, Lee SY, Chun HS. Carnosol, a component of rosemary (*Rosmarinus officinalis* L.) protects nigral dopaminergic neuronal cells. Neuroreport. 2006; 17: 1729-1733.
71. Rau O, Wurglics M, Paulke A, Zitzkowski J, Meindl N, Bock A, Dingermann T, Abdel-Tawab M, Schubert-Zsilavecz,M. Carnosic acid and carnosol, phenolic diterpene compounds of the labiate herbs rosemary and sage, are activators of the human peroxisome proliferator-activated receptor gamma. Planta Med. 2006; 72: 881-887.
72. Blumenthal M. The Complete German Commission E Monographs; American Botanical Council: Austin; 1998, p. 197.

73. Blumenthal M, Goldberg A, Brinckmann J. Herbal Medicine. Newton: Integrative Medicine Communications; 2000, pp. 326-329.
74. E/S/C/O/P Monographs. Stuttgart: Thieme; 2003, pp. 452-455.
75. Maleš Ž, Medić-Šarić M. Optimization of thin-layer chromatographic analysis of flavonoids and phenolic acids of *Salviae folium*. Acta Pharm. 1998; 48: 85-92.
76. Capek P, Hríbalová V. Water-soluble polysaccharides from *Salvia officinalis* L. possessing immunomodulatory activity. Phytochem. 2004; 65: 1983-1992.
77. Akhondzadeh S, Abbasi SH. Herbal medicine in the treatment of Alzheimer's disease. Am J Alzheimers Dis Other Demen. 2006; 21: 113-118.
78. Ninomiya K, Matsuda H, Shimoda H, Nishida N, Kasajima N, Yoshino T, Morikawa T, Yoshikawa M. Carnosic acid, a new class of lipid absorption inhibitor from sage. Bioorg Med Chem Lett. 2004; 14: 1943-1946.
79. Blumenthal M. The Complete German Commission E Monographs. Austin: American Botanical Council; 1998, p. 198.
80. Blumenthal M, Goldberg A, Brinckmann J. Herbal Medicine. Newton: Integrative Medicine Communications; 2000, pp. 330-334.
81. Hubbert M, Sievers H, Lehnfeld R, Kehrl W. Efficacy and tolerability of a spray with *Salvia officinalis* in the treatment of acute pharyngitis - a randomised, double-blind, placebo-controlled study with adaptive design and interim analysis. Eur J Med Res. 2006; 11: 20-26.
82. City Pharmacy Zagreb. [cited 2017 Jun 8]. Available: http://www.gljz.hr
83. Guaschino S, Benvenuti C. SOPHY project: an observational study of vaginal pH, lifestyle and correct intimate hygiene in women of different ages and in different physiopathological conditions. Part II. Minerva Ginecol. 2008; 60: 353-362.

Standardization and phytochemical investigations using modern analytical techniques

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ABSTRACT

Standardization of crude herbal drugs and phytochemical investigations are inevitable parts of the drug discovery process for obtaining newer drugs for the treatment of various diseases and ailments. Standardization of crude herbal drugs refers to analysing the quality and purity of the crude drugs before their further processing and remains a prerequisite step for the preparations of herbal formulations. It involves authentication of crude drugs, analysing the quality and purity of the crude drugs via several means of evaluation such as organoleptic, microscopic, chemical, physical and biological means of evaluation. However, today's era involves the standardization of crude herbal drugs via fingerprinting of herbal extracts/isolates using two techniques. The first technique involves the usage of chromatography for the purpose of fingerprinting of herbal extracts/isolates and involves several modern analytical techniques such as HPLC and LC-MS/MS whereas the second technique involves PCR-based method for carrying out DNA fingerprinting of crude herbal drugs. Phytochemical investigations also involve usage of various analytical techniques for the purpose of isolation and characterization of phytoconstituents. Primitive techniques basically involved usage of column chromatography for the purpose of isolation but today modern analytical techniques are available that are used for the isolation of active constituents

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from the herbal drugs such as modern flash chromatography, semi-preparative and preparative HPLC's, that offer faster and highly precise separation of the phytoconstituents. The present chapter entails the various fingerprinting techniques used for the standardization of crude herbal drugs and also covers the various modern techniques used for isolation of phytoconstituents.

Keywords: Standardization; Fingerprinting; Isolation; Chromatography; HPLC; LC-MS/MS; DNA.

1. INTRODUCTION

A number of approaches on drug discovery and development from traditional medicines have been practised by scientists from world over for years together. The history of traditional medicines has helped in the development of several new molecules that have implications for new drug discovery and also act as precursors for providing new chemical entities. The values of natural products have been well studied and documented by our ancestors and since time immemorial, natural products have provided a vivid opportunity for researchers to use this documented knowledge for obtaining newer leads for drug discovery. Thereof, the secondary metabolites obtained from natural resources have been perceived as showing noticeable pharmacological actions with higher potency and lesser adverse effects making them good leads for further development. The new molecules in the form of secondary metabolites evolved in the nature through plants and animals and further research on these secondary metabolites with the help of new drug discovery tools is vital for the enhancement of human health. Most of the potent phytoconstituents of today including the aspirin to taxol have been derived from plants itself and beside these thousands of other phytoconstituents with therapeutic potentials would require more sophisticated techniques of rapid investigation whether for the purpose of standardization or for the purpose of isolation [1-2]. The present chapter entails the modern analytical techniques that are being used for the standardization of herbs as well as for the isolation of phytoconstituents at semi-preparative and preparative scales.

2. STANDARDIZATION OF HERBS USING MODERN ANALYTICAL TECHNIQUES

Standardization of crude herbal drugs is an inevitable part of the drug discovery process and refers to analysing the quality and purity of the crude drugs before their further processing and remains a prerequisite step for the preparations of herbal formulations. It involves authentication of crude drugs, analysing the quality and purity of the crude drugs via several means of evaluation such as organoleptic, microscopic, chemical, physical and biological means of evaluation. Among these evaluation techniques used for standardization of crude drugs, there are various methods that have been adopted since time such as morphological and sensory evaluation, chemical tests that have used for preliminary phytochemical screening, determination of ash value, extractive value, foreign matter etc. However, today's era involves the standardization of crude herbal drugs via fingerprinting technology wherein all the phytoconstituents present in the herb are enlisted in a sequential manner depending on the technique used for the purpose of fingerprinting [3]. Fingerprinting of crude herbal drugs can be done in two ways (Figure 1). The first fingerprinting method involves usage of a chromatographic technique such as high performance liquid chromatography (HPLC), gas chromatography (GC) etc. or a chromatographic technique coupled with a analytical techniques such as liquid chromatography coupled with two mass spectrometers (LC-MS/MS) or GC coupled with mass spectroscopy (GC-MS) etc. The second method for fingerprinting involves DNA fingerprinting technique wherein the DNA analysis of the crude drug has been used for its authentication and therefore act as an important tool for herbal drug standardization [4].

2.1. Chromatographic fingerprinting of herbal extracts/isolates using modern analytical techniques

Chromatographic fingerprinting is a process by which the various constituents present in the crude herbal drugs are separated out in a single chromatogram, enabling to view precisely the entire range of the constituents present and is highly specific for a particular crude drug. Therefore, this technique is nowdays being used for standardization of crude drugs using various chromatographic techniques such as HPLC, GC etc. in alone or in combination with the advanced analytical techniques for obtaining more precise and accurate

results e.g. chromatographic fingerprinting of crude drugs using liquid chromatography tandem mass spectroscopy (LC-MS/MS) or GC-MS etc.

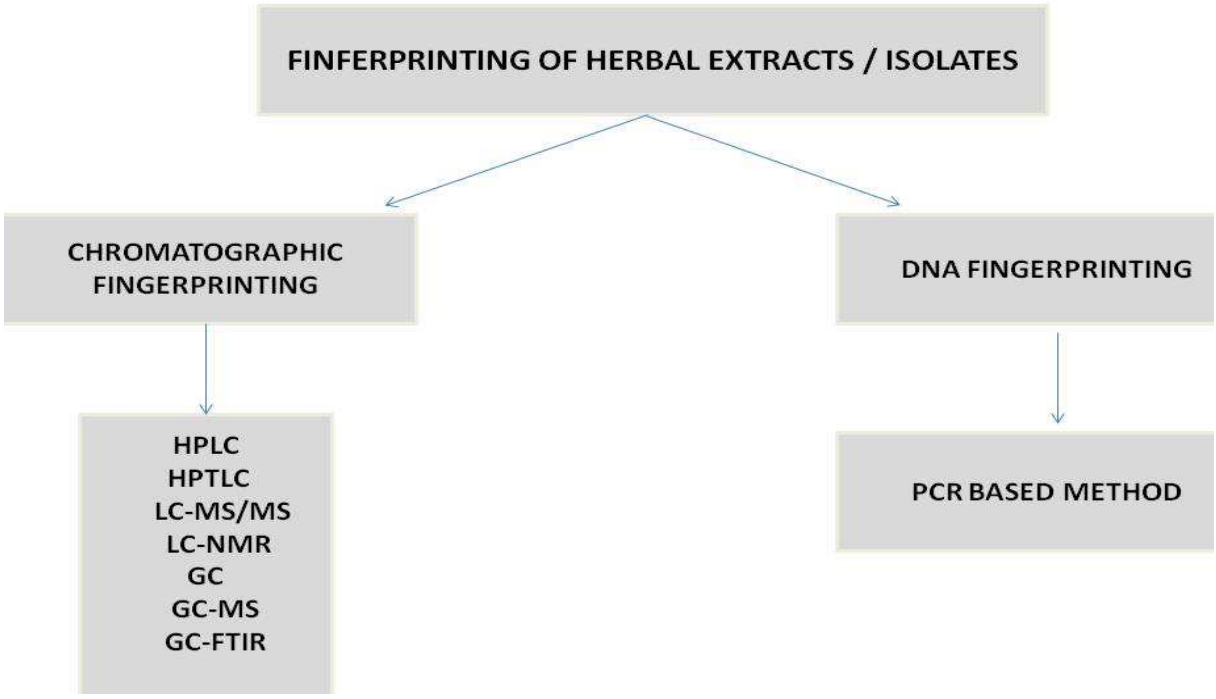

Figure 1. Methods for fingerprinting herbal extracts/isolates

These techniques not only provide precise fingerprint spectra of the entire range of constituents present in the herb but also provide additional information's related to the characteristics of the phytoconstituents e.g. fingerprinting via LC-MS/MS provides molecular weight of the constituents, provides information regarding the fragmentation pattern of the individual phytoconstituents and also provides information related to the fragmentation pattern of the daughter nuclei that are generated from the parent nuclei giving a sound base for the structure elucidation of the phytoconstituents.

2.1.1. Chromatographic fingerprinting of herbal extracts/isolates using HPLC

The chromatographic fingerprinting of plant extracts using HPLC is one of the commonly used methods for identifying the phytoconstituents present in the crude herbal extract [5]. In principle, the herbal extract is weighed and re-constituted in an appropriate vehicle to develop a solution of known strength. Appropriate dilutions of the sample are

analyzed using HPLC. Under standardized conditions of HPLC, the sample is pressurized along with the mobile phase through the column to separate it into bands which are eluted, detected and recorded. The system offers numerous advantages like: rapid separation, high resolution, detection upto ng quantities of sample, continuous monitoring using ultraviolet or fluorescence detectors and easy coupling with spectrometers such as mass spectrometer and nuclear magnetic resonance spectrometer. It therefore, provides an ideal setting for separating meager quantities of structurally related mixture of compounds from natural sources. The chromatogram is now known as the "fingerprint" of the sample (Figure 2). It is matched each time whenever fresh extraction is carried out. This helps to maintain the chemical quality of the plant extracts as the observations will reveal the fact that whether the same species have been used everytime for extraction or not. If the plant species used for the extraction is different or there is higher adulateration in case of the crude drug, then the chromatographic spectra obtained using HPLC will differ noticeably each time the fingerprinting is repeated for the same species. Thus, the most factual point is this technique is that no two plant species will show the same chromatographic spectra in HPLC.

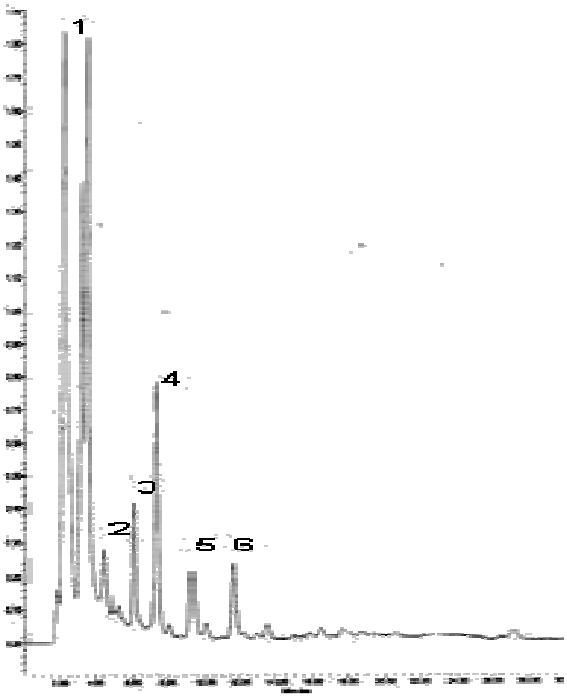

Figure 2. Fingerprint chromatogram of crude herbal extract showing presence of various phytoconstituents present (peaks 1-6)

2.1.2. Chromatographic fingerprinting of plant extracts using HPTLC

High performance thin layer chromatography (HPTLC) is another chromatographic technique that can be used to develop fingerprint chromatogram of the plant extracts/isolates under standardized conditions [6]. HPTLC is a semi-automatic thin layer chromatography technique wherein the optical density (absorption) of the separated spots are measured in addition to the rentention factor (R_f) values. This technique is now being used for the fingerprinting of the phytoconstituents present in the plant extracts that can be matched batch to batch so as to ensure accuracy in results for the purpose of quality control of herbal drugs.

2.1.3. Chromatographic fingerprinting of herbal extracts/isolates using LC-MS/MS

Liquid chromatography tandem mass spectroscopy (LC-MS/MS) is an advanced analytical technique that finds tremendous application in the standardization of crude herbal drugs. The technique involves coupling of HPLC with two mass spectrometers (thereby known as LC-MS/MS) and is being used for the fingerprinting of crude herbal extracts/isolates [7]. LC-MS/MS involves two mass spectrometers wherein the first one depicts the molecular weights of the phytoconstituents (also called as parent ions) present in the crude drug extract while the second mass spectrometer depicts the molecular weights of the daughter ions. This technique is helpful in determining the fragmentation patterns of the parent compounds by depicting all the daughter fragments that are formed from an individual parent compound. The fragmentation pattern of the parent ions and the daughter ions are unique and the information enables the user to understand the mechanism as to how the parent and daughter ions are getting fragmented. Thus, the technique enables convenience in differentiation of crude herbal drug from various other similar species and also from adulterants and problem of quality assurance of herbal drugs can at large be solved with the use of LC-MS/MS fingerprinting technique.

The fingerprinting methodolgy involves subjecting the crude drug extract for gradient separation using electrospray ionization (ESI) mode in LC-MS/MS. The chromatographic separation of the plant extract can be achieved using C_{18} column using a linear gradient mobile phase reaching 90% acetonitrile (with 0.1% formic acid) from 10% over the period of 30 mins with mobile phase setted at the rate of 0.5 ml/min. Source dependent parameters such as Gas 1 and Gas 2 can be standardized by keeping at 30 psi and 60 psi at the ESI temperature of 450°C. The information dependent aquisition (IDA) protocol consisting of enhanced mass

spectrometry (EMS), enhanced resolution (ER) and enhanced product ion scan (EPI) can be performed at the speed of 4000 Da/sec between 100-800 amu at the positive ESI (+5500 V) temperature of 450°C (Figure 3).

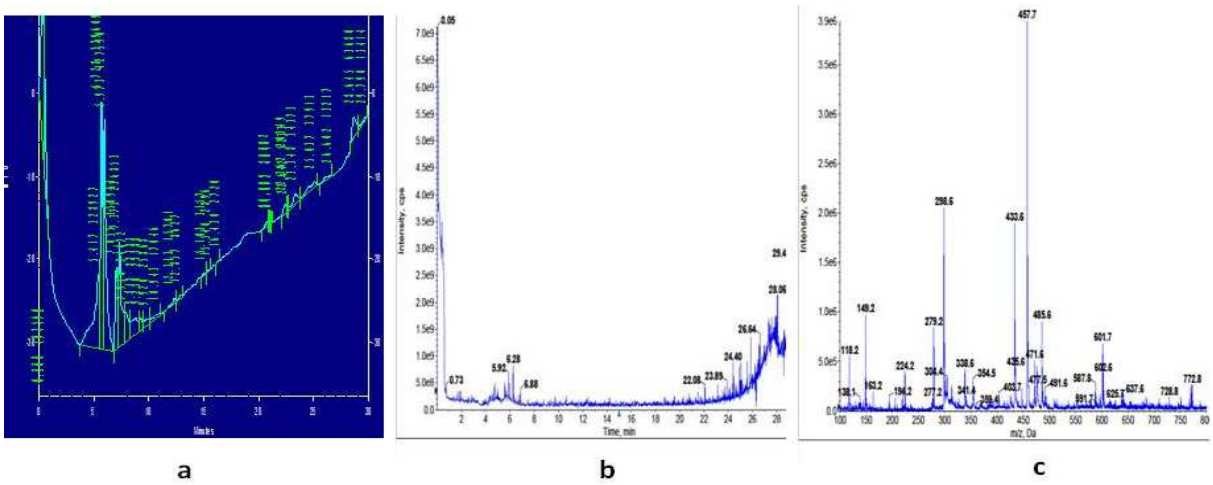

Figure 3. LC-MS/MS scan of crude herbal extract: (a) LC scan, (b) enhanced mass spectra and (c) enhanced resolution

Post acquisition of data, UV photodiode array spectrum guided analysis of secondary metabolite can be conducted in the EMS scan of the extract at the same retention time. The unprotonated molecular weight matching the known mass values of the secondary metabolites library can be subjected for fragment analysis using inbuilt algorithm for fragment interpretation tool. The identified secondary metabolites can be verified and confirmed using product ion spectrum using flow infusion analysis.

2.1.4. Chromatographic fingerprinting of herbal extracts/isolates using LC-NMR

The liquid chromatography coupled with nuclear magnetic resonance spectroscopy (LC-NMR) is another advantageous technique used for the chromatographic fingerprinting of crude drug extracts as it offers precise fingerprint spectra of the crude herbal extract in relatively lesser time period. The LC-NMR technique allows the continuous registration of variability in the proton spectra of the phytoconstituents as they appear in the chromatogram. Also, the automated data acquisition and processing technology in LC-NMR improve the speed and sensitivity of detection of phytoconstituents [8].

2.1.5. Chromatographic fingerprinting of herbal extracts/isolates using GC

Gas chromatography is another important technique that can be used for the chromatographic fingerprinting of phytoconstituents particularly those that are volatile in nature and therefore is used for the authentication and quality control of crude herbal drugs. The high selectivity of capillary columns enables separation of many volatile compounds simultaneously within comparatively shorter time span [9]. However, this technique cannot be used for crude drug samples wherein the phytoconstituents are thermolabile and non-volatile.

2.1.6. Chromatographic fingerprinting of herbal extracts/isolates using GC-MS

Gas chromatography coulped with mass spectroscopy (GC-MS) has been used for the fingerprinting of phytoconstituents that are volatile in nature [10]. The system offers higher sensitivity, stability and efficiency of chromatographic separation which is based on the molecular weight of the phytoconstituents. The simplest mass detector in the instrument is the ion trap detector (ITD). In this instrument, ions are created from the eluted sample by electron impact or chemical ionization and stored in a radio frequency field, the trapped ions are then ejected from the storage area to an electron multiplier detector. The ejection is controlled so that scanning on the basis of mass to charge ratio is possible.

2.1.7. Chromatographic fingerprinting of herbal extracts/isolates using GC-FTIR

Gas chromatography coulped with Fourier transform infrared spectroscopy (GC-FTIR) is another useful technique that provides a potent means of fingerprinting phytoconstituents especially of volatile nature [11].

2.2. Fingerprinting of crude herbal drugs using DNA technology

Among the newer methodologies, DNA fingerprinting is one such technique which has evolved with time and has been found to possess tremendous potential for the standardization of crude drugs [12]. In this technique, the DNA analysis of the crude drug has been used for its authentication and for the determination of its quality and purity. This technique also finds application in differentiating the herbal drug from various other adulterants or substituted drugs and offers unique advantage as the DNA fingerprint genome

for any crude drug remains same irrespective of the part of the plant that has been used for extraction. Thus, the problem of quality assurance of herbal drugs can at large be solved with the use of DNA fingerprinting technique. The most commonly used methodology engages polymerase chain reaction (PCR) - based method for the purpose of standardization of crude herbal drugs.

2.2.1. Fingerprinting of crude herbal drugs using polymerase chain reaction (PCR) - based method

The polymerase chain reaction (PCR) is a biochemical technology in molecular biology that has beeen used nowdays for the fingerprinting of crude herbal drugs. It is used to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence [13]. Primers, origional DNA (extracted from the plant cell) which is to be amplified, a specific type of DNA polymerase and the necessary chemicals for DNA synthesis are mixed. Briefly, the steps involved in the process are:

1. **Initialization step**: This step consists of heating the reaction to a temperature of 94-98°C (depending upon the polymerase used) and is mainly used for polymerases that require heat activation by hot-start PCR.
2. **Denaturation step**: This step is the first regular cycling event and consists of heating the reaction to 94-98°C for 20-30 sec. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complimenatry bases yielding single stranded DNA molecules.
3. **Annealing step**: The reaction temperature is lowered to 50-65°C for 20-40 sec allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5°C. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation.
4. **Extension/Elongation step**: The temperature at this step depends on the DNA polymerase used e.g. Taq polymerase has its optimum activity temperature at 75-80°C. At this step the DNA polymerase synthesizes a new DNA strand complimentary to the DNA template strand by adding dNTPs and the extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified.
5. **Final Elongation step**: This single step is ocassionally performed at a temperature of

70-74°C for 5-15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

6. Final Hold: This step may be employed for short term storage of the reaction at 4-15°C.

3. PHYTOCHEMICAL INVESTIGATIONS OF CRUDE HERBAL EXTRACTS USING MODERN ANALYTICAL TECHNIQUES

Phytochemical investigations of crude herbal extracts remain a mainstay in drug discovery for obtaining newer compounds with potent pharmacological actions and involve usage of various chromatographic and analytical techniques for the purpose of isolation and characterization of phytoconstituents. Primitive techniques basically involved usage of column chromatography for the purpose of isolation but today modern analytical techniques are available that are used for the isolation of active constituents from the herbal drugs such as semi-preparative HPLC, preparative HPLC, modern flash chromatography etc. These techniques provide several advantages over the primitive techniques as they offer faster and precise separation of the phytoconstituents and serve as a source for obtaining highly purified compounds especially in large quantities.

Column chromatography was the most commonly used primitive technique for the isolation of phytoconstituents at the preparative scale and this technique offered several advantages such as simple packing procedures, low operating pressure and low expense for instrumentation [14]. Column chromatography was later divided into two categories depending on the way the solvent flowed down the column. The first category was gravity column chromatography wherein the solvent flows down the column by gravity and the second category was known as flash chromatography where the solvent was forced down the column by positive air pressure. In the traditional column chromatography, also called as gravity column chromatography, the extract to be isolated was placed on the top of the column containing some solid support, often silica gel. The rest of the column was then filled with a solvent (in a sequential manner depending on the polarity or the type of constituent to be isolated) which then runs through the solid support under the force of gravity. The various phytoconstituents to be isolated travel through the column at different rates and then can be collected separately as they emerge from the bottom of the column. However, this technique was time consuming as the rate at which the solvent flows down through the column was slow. The flash chromatography, also known as medium pressure chromatography was

introduced by Clark Still in 1978 and was found to act as an alternative to the gravity fed chromatography and differed from the latter in many ways: firstly smaller silica gel particles (250-400 mesh) were used and secondly due to restricted flow of solvent caused by the smaller gel particles, pressurized gas (10-15 psi) was used to drive the solvent through the column of the stationary phase. The net result was a rapid ("over in a flash") and high resolution chromatography [15]. However, nowadays, advancements in the flash chromatography system are available with higher automation that separate the phytoconstituents from the extract from a few milligrams up to kg scale and offer cheaper and quicker solution for carrying out isolation of phytoconstituents.

3.1. Modern flash chromatography for isolating phytoconstituents at preparative scale

Modern flash chromatography is a modern reverse phase column chromatographic technique used for carrying out isolation of phytoconstituents from crude drug extracts at preparative scale. The system works on the principle of adsorption chromatography. As each phytoconstituents in the extract exhibits different affinity for stationary phase, under appropriate mobile phase and ideal chromatography conditions, they elute in succession and are thus separated out. The system is designed in such a way that large quantities of sample (Kg) can be loaded and the yield of isolates can be in "mg" quantities. This technique is highly advantageous in case of isolation of phytoconstituents as these phytoconstituents are present in very low quantities in the plant and repeated separations need to be carried out for obtaining sufficient amounts of isolates. Automated flash chromatography overcame the problems that were associated with the primitive isolation technique such as time consumption for carrying out isolation and most importantly the variations in the quality of the plant isolates, thereby ensuring optimum isolation of phytoconstituents at preparative scale.

Briefly, the modern flash chromatographic system consisted of nitrogen cylinder for pressurizing (A), solvent reservoir (B), sample column (E), separating column (F) and fraction collection (G) (Figure 4). Usually, the solvent reservoir (B) is fabricated from stainless steel and has a capacity to hold large quantities of solvent. Double walled, braded tubing is used to connect nitrogen cylinder to solvent reservoir. Two high pressure ball valves help to regulate the two outlets from the solvent reservoir (C, D). The outlets are made from stainless steel and are used for the purpose of carrying pressurized solvent from the reservoir either to sample column (E) or to the separating column (F). The sample column (E) is a hollow column

wherein the sample is adsorbed on silica (mesh 200-400) and tightly packed within the sample column, whose ends are finally closed with frets. The amount of crude drug extract that can be used in a single run depends on the size of the sample column. The separating column (F) is fabricated from stainless steel and is packed with derivatized-reverse-phase silica powder. A steel outlet from this chamber helps to collect the fractions eluted.

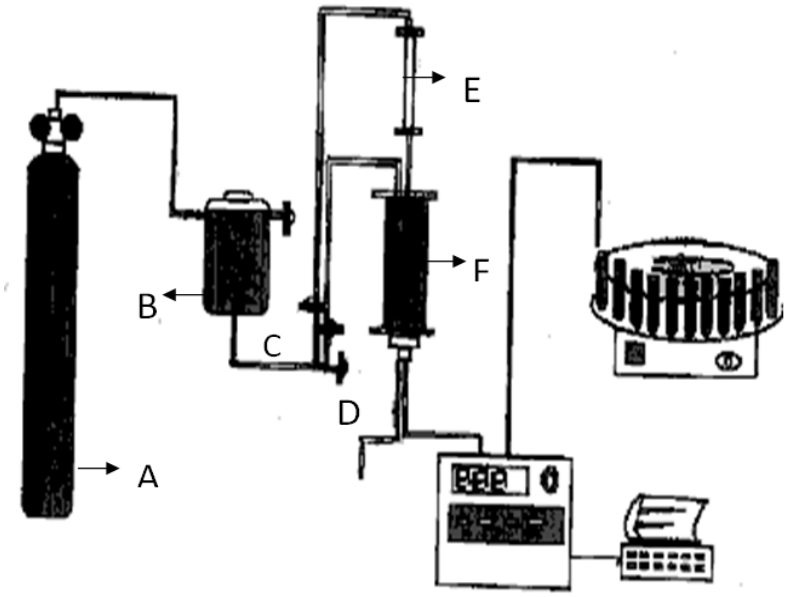

Figure 4. Modern flash chromatography set up. (A) - Nitrogen cylinder, (B) - Solvent reservoir, (C, D) - High pressure ball valves, (E) - Sample column, (F) - Separating column, (G) - Fraction collection

3.2. Isolation of phytoconstituents using semipreparative HPLC

The modern semipreparative HPLC enables the users to isolate µg quantities of plant components by using binary gradient mode [16]. Briefly, two HPLC pumps are attached via gradient controllers to maintain a flow upto 10 ml per minutes. Conventionally, methanol and water are used in decreasing polarity over a period of 10-60 minutes. Concentrated methanolic extract is injected into the system using rheodyne injector attached with a 50 µg loop. This unit is attached with a high flow cell detector having rugged optics to withstand the flow rate of 10 ml/min. The columns used for semipreparative isolation are available commercially to maintain such flow rates. With the present technology of monolithic rod columns, such high flow rates can be achieved with comparatively low back pressure.

Typically, a 10 cm × 1 cm rod column is operatable up to the pressure of 1500 psi. Miniature flash run C18 columns are also available to be used in HPLCs with PDA detector to optimize the run and to analyze the peak of interest using low flow volumes with analytical separation using gradient flow conditions.

3.3. Isolation of plant components using preparative HPLC

The modern preparative HPLC enables the users to fractionate several grams of isolates present in the crude drug extract. The methodology is same as applicable for semi-preparative HPLC, however, the columns used are bigger in sizes in comparative to the semi-prepative one and the sample ports can accomodate higher amounts of crude herbal extracts. The columns used for preparative HPLC offer low back pressure and enable larger flow rates of the mobile phases for faster seperation and higher yield of phytoconstituents.

4. SUMMARY

The problem of quality assurance in case of crude herbal drugs can be resolved to a great exteant with the help of fingerprinting techniques as the quality grade could be determined successfully using the fingerprint spectra of crude herbal drugs. The variation determination of common peaks, noticeable in the chromatographic fingerprint spectra could provide qualitative as well as quantitative information related to the phytoconstituents present in the crude herbal drugs. Pattern recognition of the chromatographic fingerprint spectra can be used for discrimination of different kind of sample of crude herbal drugs. Thus, chromatographic fingerprinting serves as a promising quality control tool for crude herbal drugs. DNA fingerprinting is another technique that has proven to be a promising tool for the standardization and quality control of crude herbal drugs. The phytochemical investigations using modern analytical techniques offer faster and precise separation of phytoconstituents and serve as a means of obtaining highly purified phytoconstituents especially in larger quantities, that may act as newer drugs with potent pharmacological actions.

REFERENCES

1. Norn S, Permin H, Kruse PR, Kruse E. From willow bark to acetylsalicylic acid. Dan Medicinhist Arbog. 2009; 37: 79-98.

2. Adams JD, Flora KP, Goldspiel BR, Wilson JW, Arbuck SG, Finley R. Taxol: a history of pharmaceutical development and current pharmaceutical concerns. J Natl Cancer Inst Monogr. 1993; 15: 141-147.
3. Gupta P. DNA and LC-MS/MS fingerprintings as novel tools for the standardization of crude herbal drugs. Nat Prod Chem Res. 2015; 3: 3.
4. Kulkarni KM, Patil LS, Khanvilkar VV, Kadam VJ. Fingerprinting techniques in herbal standardization. Indo Am J Pharm Res. 2014; 4(2): 1049-1062.
5. Kalpana MJ, Sowjanya GN, Ajitha A, Rao VUM. Fingerprint analysis of herbal medicines using HPLC: an overview. Int J Pharmac Res Anal. 2014; 4(4): 250-259.
6. Rasheed A, Sravya B, Roja C. Review on standardization of herbal forulations. Int J Phytother. 2012; 2(2): 74-88.
7. Velpandian T, Gupta P, Ravi AK, Sharma HP, Biswas NR. Evaluation of pharmacological activities and assessment of intraocular penetration of an ayurvedic polyherbal eye drop (Itone^{TM}) in experimental models. BMC Complim Altern Med. 2013; 13: 1-12.
8. Liang Y, Xie P, Chan K. Quality control of herbal medicines. J Chromatogr B Analyt Technol Biomed Life Sc. 2004; 812(1-2): 53-70.
9. Nikam P, Kareparamban J, Jadhav A, Kadam V. Future trends in standardization of herbal drugs. J Appl Pharmac Sci. 2012; 2(6): 38-44.
10. Patel P, Patel N, Goyal R. Quality control of herbal products. Indian Pharmacist. 2006; 5(45): 26-30.
11. Yadav N, Dixit V. Recent approaches in herbal drug standardization. Int J Integr Biol. 2008; 2(3): 196.
12. Choudhary N, Sekhon B. An overview of advances in standardization of herbal drugs. J Pharm Educ Res. 2011; 2(2): 55-64.
13. Lin CH, Chen BH. Determination of carotenoids in tomato juice by liquid chrpmatography. J Chromatogr A. 2003; 1012(1): 103-109.
14. Clark Still W, Kahn M, Mitra A. Rapid chromatographic technique for preparative separations with moderate resolutions. J Org Chem. 1978; 43(14): 2923-2925.
15. Ayare P, Khan Vilkar V, Chalak N. Flash chromatography: area and applications. Pharma Tutor. 2014; 2(5): 89-103.
16. Sharma U, Velpandian T, Sharma P, Singh S. Evaluation of anti-leishmanial activity of selected Indian plants known to have antimicrobial properties. Parasitol Res. 2009; 105(5): 1287-1293.

Drimane sesquiterpenes from *Drimys winteri*

6

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ABSTRACT

Canelo (*Drimys winteri* J.R., Winteraceae) is a native tree of center-south of Chile, which is sacred for the *Mapuche*, the native people of this area. Thirteen drimane sesquiterpenoids have been reported as main secondary metabolites from leaves and bark of *D. winteri*. Their biological activities range from the activation of calcium ion channels to antimicrobial and antiparasite activity. The drimane sesquiterpenoids share a *trans*-fused decaline skeleton with an angular methyl group at C-10 and a *gem*-dimethylgroup at C-4. This chapter highlights procedures for their isolation from the natural source, the structure elucidation using high-field NMR-methods, including 2D-NMR-spectroscopy, their biosynthesis and bioactivities as well as strategies for their chemical synthesis.

Keywords: Drimane Sesquiterpene; *Drimys winteri*; Synthesis; 1D and 2DNMR; Bioactivities.

1. INTRODUCTION

Drimys winteri J.R. (Winteraceae), usually called Canelo, is a native tree of center-south of Chile and Argentina. It is found up to 1,200 m above sea level and between latitude

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32° south and Cape Horn at latitude 56° in Tierra del Fuego, Patagonia. The plant was described by Johann Reinhold Forster and his son Johann Georg Adam Forster in 1775 [1]. *Drimys winteri* J.R. is an evergreen tree which grows up to 20 m in height and can tolerate temperatures till -20°C. The leaves are lanceolate, glossy green on the top face and whitish below, and can measure up to 20 cm. The flowers are white with a yellow center and consist of a great number of petals and stamens.

The native people of Chile called *Mapuche* consider this tree sacred, a symbol of benevolence, peace and justice. It is present in all social and religious meetings called "guillatún" and "machitún" where the healer or "machi" uses leaves or sap of Canelo as medicine [2].

The main secondary metabolites in Canelo are drimane sesquiterpenoids which were described by H.H. Appel [3-5]. The name *drimane* is derived from the sesquiterpenoid alcohol drimenol (**2**) and describes the saturated hydrocarbon decalin moiety with the structure and absolute configuration shown in structure **1** in Fig. 1. These compounds were reviewed by B. Jansen (2004) [6].

Drimys winteri J.R. belongs to the *Canellaceae* family and the genus *Winterana*, other genera are *Cinnamodendron* endemic to South America, *Warburgia* to South East Africa and *Cinnamosma* to Madagascar. From all constituent species drimane sesquiterpenoids with medicinal properties, such us fungicidal, insecticidal, antiinflamatory and bactericidal properties were reported. These metabolites are also useful in the chemotaxonomy of the family [7].

2. BIOSYNTHESIS AND BIOTRANSFORMATION OF DRIMANE SESQUITERPENOIDS

Drimane sesquiterpenoids are biosynthesized from farnesylpyrophosphate (FPP), through a process which is initiated by protonation of the double bond at the head position of FPP, initializing a double cyclization process, which yields drimenyl pyrophosphate with the A,B ring system similar to many triterpenoids [8]. Hydrolysis of this intermediate gives drimenol (**2**). The stereochemistry of the final product is determined by the orientation of the substituents and the double bonds in the chair-like conformation of the FPP chain prior to cyclization (Scheme 1). Further enzymatic oxidations of drimenol lead to a broad number of compounds shown in Fig. 1.

The enantioselective activation of a sp^3 carbon is a difficult task in organic synthesis due to the high energy required for activation of C-H and C-C bonds. In the drimane skeleton the activation of position 3 has been reported by microbial oxidation to yield 3β-hydroxy drimane derivatives [9].

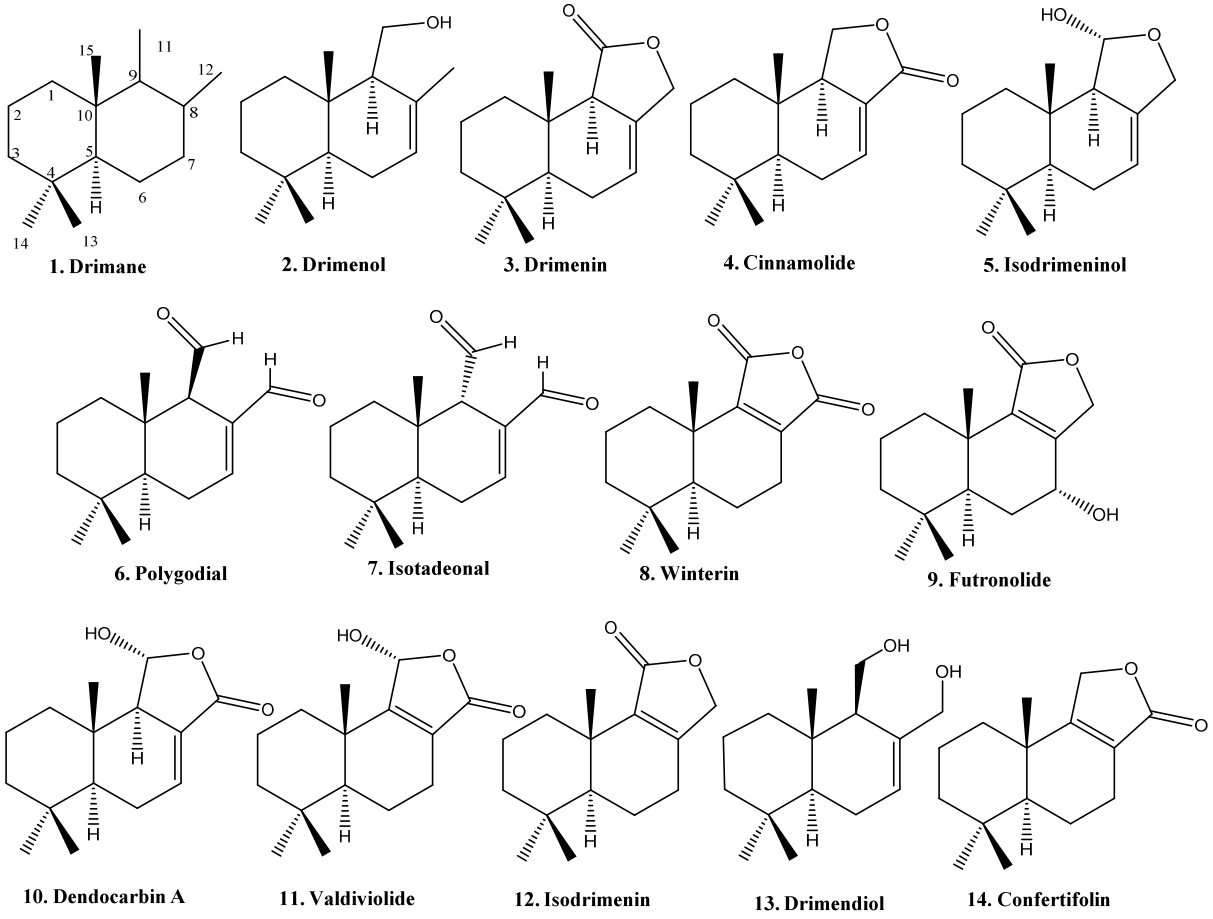
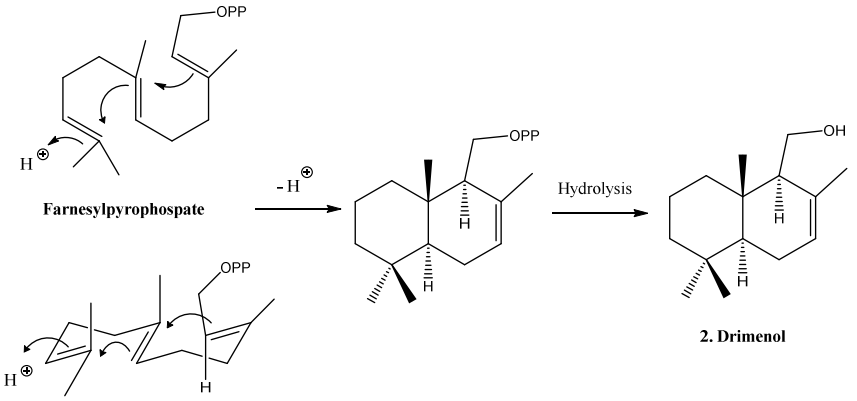


Figure 1. Structures of natural drimane sesquiterpenoids isolated from *D. winteri*



Scheme 1. Biosynthesis of drimenol

Incubation of confertifolin (**14**) at a concentration of 0.5 g/l with *Mucor plumbeus*, *Aspergillus niger* or *Rhizopus arrhizus* for 48 h gave, independent of the microorganism, the corresponding 3β-hydroxyconfertifolin in good yields, while the same experiment with isodrimenin (**12**) incubated with *A. niger* or *M. plumbeus* afforded a major metabolite characterized as 3 β-hydroxyisodrimenin. However, on incubation with *R. arrhizus*, isodrimenin afforded two isomeric hydroxylated metabolites, one of them being identical to 3β-hydroxyisodrimenin but the other compound was the known natural 7a-hydroxy derivative futronolide (**9**) [10].

3. PURIFICATION AND NMR CHARACTERIZATION OF DRIMANE SESQUITERPENOIDS FROM *DRIMYS WINTERI*

Barks of *Drimys winteri* are suitable for the extraction of drimane sesquiterpenoids, which must be crushed and extracted by maceration with organic solvents such us light petroleum, dichloromethane, chloroform or ethyl acetate. Considering that drimane compounds are mostly lipophilic, good extractions were performed with ethyl acetate or ethanol for 3 days at room temperature without using halogenated solvents. The organic layer is evaporated *in vacuo* giving a crude gummy oil which can be fractionated by distillation at 10^{-4} mmHg and temperatures between 130 to 260°C. The fractions must be further purified by column chromatography. According to this protocol, confertifolin (**14**), valdiviolide (**11**) and futronolide (**9**) were isolated [5]. As the high temperatures required for distillation can destroy the sensitive compounds, the crude total extract can be purified by column chromatography on silica gel using solvent mixtures of increasing polarity (e. g. hexane/ethyl acetate). Considering that these compounds are not highly polar, the major compounds can be eluted with a hexane/ethyl acetate (4:1 v/v) solvent mixture. However, it is very important to start the chromatography with pure hexane, because the extract contains ca. 40% of fatty acids and aromatic small molecules which could interfere with the purification of drimanes.

The chromatographic purification is monitored by thin-layer chromatography (TLC). Classical developer reagents are useful such as UV-light (220 nm, nondestructive technique) followed by H_2SO_4 (30 wt-% in water), which is sprayed over the TLC plate and then heated. Most of the drimanes can be detected with H_2SO_4, with the exception of cinnamolide (**4**) which is detected with UV-light.

With hexane/ethyl acetate (9:1 (v/v)) as eluent, the compounds were eluted in the order drimenin (**3**) (colorless crystals), drimenol (**2**) (colorless crystals), cinnamolide (**4**) (colorless crystals), isodrimenin (**12**) (colorless crystals) and confertifolin (**14**) (colorless crystals). Eluting with hexane/ethyl acetate (4:1 (v/v)) gave isodrimeninol (**5**) (yellow oil), isotadeonal (**7**) (yellow oil) and polygodial (**6**) (yellow oil). With hexane/ethyl acetate (1:1 (v/v)) valdiviolide (**11**) (colorless crystals) and dendocarbin A (**10**) (colorless crystals) were obtained, and elution with hexane/ethyl acetate (2:3 (v/v)) furnished futronolide (**9**) (colorless crystals) and winterin (**8**) (colorless crystals) [5, 11].

The pure compounds are usually colorless crystals with the exception of isodrimeninol (**5**), polygodial (**6**) and isotadeonal (**7**), which are oils at room temperature. The structures of some drimanes have been confirmed by X-ray crystallography studies [12-15] where a basic structural framework is shown. To a *trans*-decalin moiety usually a lactone is annellated at the C-11-C-12 bond. Either the C7-C8 bond or the C-8-C-9 bond may be unsaturated, resulting in a twisted chair conformation of the second ring.

The structure elucidation of drimanes is possible by high field NMR spectroscopy [16-19]. Most naturally occurring drimanes have a carbonyl group either at C-11 or at C-12 and at least one endocyclic C-C-double bond. The ^{13}C-NMR-spectrum can provide valuable information in this regard: for example, a resonance at 170 ppm is generally indicative for a lactone structure. The two aldehyde groups present in polygodial can be distinguished by their chemical shift values at 202 ppm (for C-11, isolated carbonyl carbon) and 193 ppm (for C-12), which appears at higher field due to conjugation with the C-7-C-8 double bond. The signals for C-7 and C-8 are found at 154 ppm and 138 ppm, respectively. The comparatively large difference of the chemical shift values for C-7 and C-8 is another indicator for the conjugation with the carbonyl carbon C-12. An equally useful indicator to determine whether a C-C-double bond is in conjugation with a carbonyl carbon are the chemical shift values of the olefinic protons in the ^{1}H-NMR-spectra (Table 1).

For drimenol (**13**) and drimenin (**3**), with an isolated C-C-double bond, the $\delta(^{1}H)$ value of H-7 is between 5.5 and 6.0 ppm, whereas this value is shifted downfield to 6.8-7.1 ppm for cinnamolide (**4**), dendocarbin A (**10**) and polygodial (**6**). If no vinylic protons appear in the ^{1}H-NMR-spectrum, but signals from quaternary carbons are found in the olefinic region of the ^{13}C-NMR-spectrum, the C-C-double bond is located between C-8 and C-9.

In Fig. 2, the ^{1}H-NMR spectrum of drimenin (**3**) is presented. Note that both protons H-12 are diasterotopic but overlap at 4.64 ppm. In Fig. 3, the ^{1}H-NMR spectrum of isodrimeninol (**5**) is shown ($CDCl_3$, singlet at 7.26 ppm originates from residual $CHCl_3$).

Table 1. [1]H-NMR (600 MHz) results for drimane sesquiterpenoids purified from *D. winteri*

H	Drimenol[a]	Drimenin[a]	Cinnamolide[a]	Dendocarbin A[b]	Polygodial[a]
1	1.06, td, J = 13.1, 3.7 1.95, ov	1.22, *m* 2.49, *dq*, *J* = 13.4, 2.7	1.20, *m* 1.59, *m*	1.30, *m* 1.81, *m*	1.39, *td*, *J* = 13.2, 2.5 1.84, *dq*, *J* = 13.2, 2.5
2	1.45, ov 1.56, qt, J = 13.6, 3.1	1.49, *m* 1.59, *dq*, *J* = 13.7, 3.2	1.49, *m* 1.59, *m*	1.50, *m* 1.60, *m*	1.52, 2H, *m*
3	1.42, ov 1.18, ov	1.49, *m* 1.24, *m*	1.25, *m* 1.51, *m*	1.50, *m* 1.25, *m*	1.49, *m* 1.24, *m*
5	1.17, ov	1.36, *dd*, *J*= 11.7,5.3	1.39, *dd*, *J* = 11.6, 5.3	1.36, *dd*, *J* = 11.7, 5.3	1.26, *m*
6	1.98, ov 1.86, ov	2.21, *m* 1.98, *m*	2.41, *ddd*, *J*= 20.3, 8.9, 3.7 2.11, *dddd*, *J* = 20.2, 11.8, 5.0, 3.3	2.41, *dqd*, *J* =20.2, 4.3, 0.5 2.09, *dddd*, *J* = 20.2, 11.8, 4.7, 3.3	2.51, *ddddd*, *J* = 20.3, 5.5, 4.5, 2.1, 0.8 2.32, *dddd*, *J* = 20.4, 12.1, 3.9, 2.4
7	5.54, m	5.74, br *s*	6.88, *q*, *J* = 3.5	6.82, *m*	7.14, *dt*, *J* = 5.0, 2.2
9	1.84, ov	2.78,br *s*	2.82, *m*	2.52, *m*	2.83, *ddd*, *J* = 6.1, 4.1, 2.1
11	3.85, dd, J = 11.3, 3.4 3.73, dd, J = 11.3, 5.0	-	4.04, *t*, *J* = 9.1 4.38, *t*, *J* = 9.2	5.64, *br d*, J = 6.0	9.53, *d*, *J* = 4.4
12	1.78, 3H, s	4.66, 2H, *m*	-	-	9.46, *s*
13	0.86, 3H, s	0.90, *s*	0.92, *s*	0.91, *s*	0.92, *s*
14	0.88, 3H, s	0.92, *s*	0.95, *s*	0.93, *s*	0.96, *s*
15	0.85, 3H, s	0.88, *s*	0.81, *s*	0.85, *s*	0.95, *s*

[a] in $CDCl_3$. [b] in CD_2Cl_2.δ in ppm. *J* in Hz

The signal at 3.28 ppm originates from the interchangeable OH-proton. The double set of signals is caused by the presence of the C-11 anomer.

Table 2. ^{13}C-NMR (150 MHz) results for drimane sesquiterpenoids purified from *D. winteri*

C	Drimenol[a]	Drimenin[a]	Cinnamolide[a]	Dendocarbin A[b]	Polygodial[a]
1	40.0	38.4	39.5	39.4	39.5
2	18.7	18.2	18.2	18.5	18.0
3	42.2	42.3	42.2	42.2	41.7
4	33.1	33.0	32.8	33.0[c]	33.1
5	50.0	49.6	49.6	49.4	48.9
6	23.7	23.3	24.9	25.1	25.2
7	124.3	121.2	136.5	137.3	154.3
8	133.0	129.8	127.1	128.2	138.2
9	57.4	53.6	50.8	59.4	60.3
10	36.1	34.3	34.2	34.3	36.8
11	61.1	175.4	67.3	99.4	202.0
12	22.1	69.8	170.4	168.2	193.2
13	33.5	33.0	33.1	33.1[c]	33.1
14	22.2	21.4	21.3	21.3	22.9
15	15.1	13.9	13.4	14.5	15.2

[a] in $CDCl_3$. [b] in CD_2Cl_2.δ in ppm

PROBLEM 1.

The 1D and 2D NMR-spectra depicted in Figures 4-7 were recorded for one of the drimane sesquiterpenes shown in Figure 1. As an exercise, the readers are encouraged to propose the structure of this unknown drimane, based on the spectra provided! Solution of the problem is at the end of chapter.

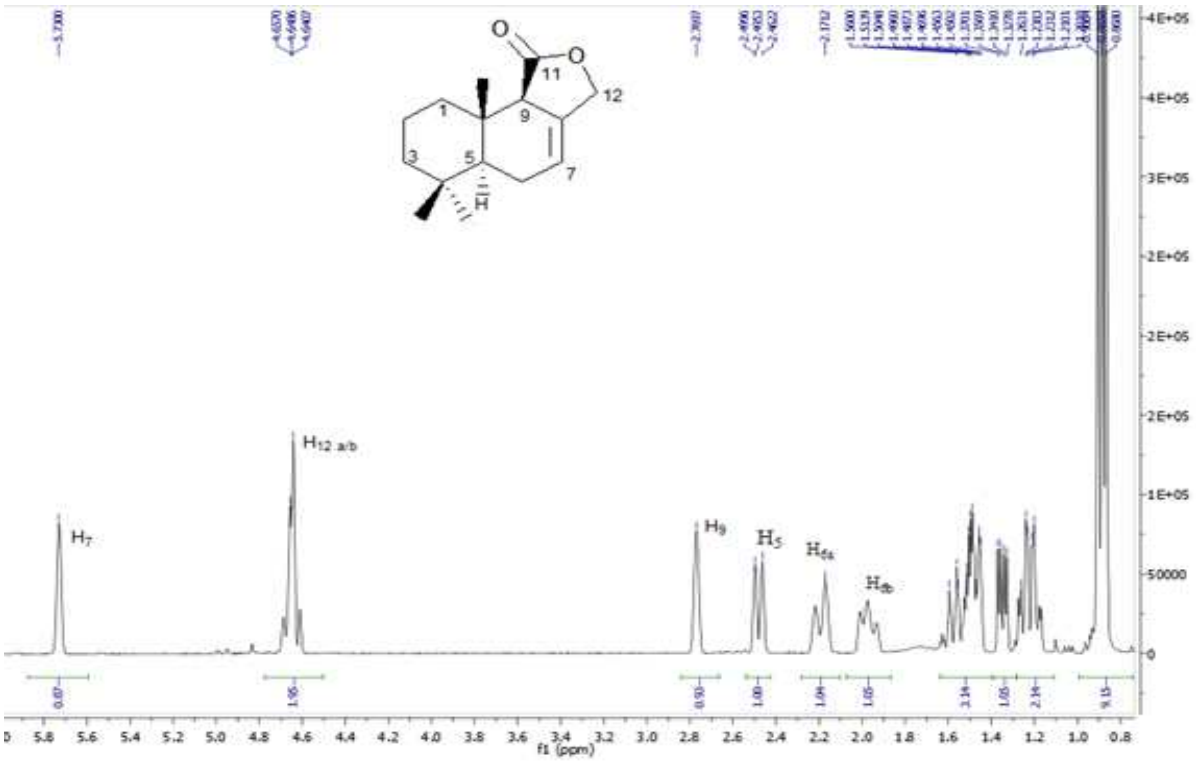


Figure 2. ^{1}H NMR (400 MHz, $CDCl_3$) of drimenin

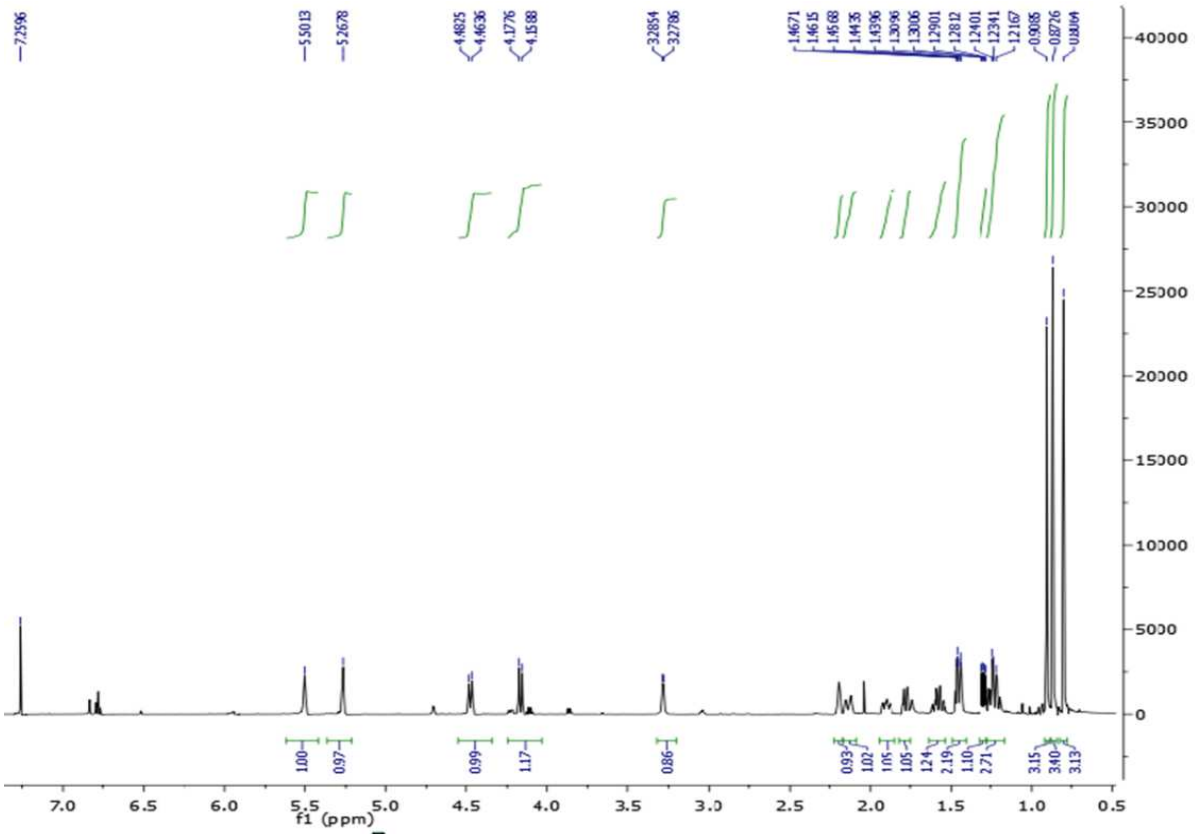


Figure 3. ^{1}H NMR (400 MHz, $CDCl_3$) of isodrimeninol

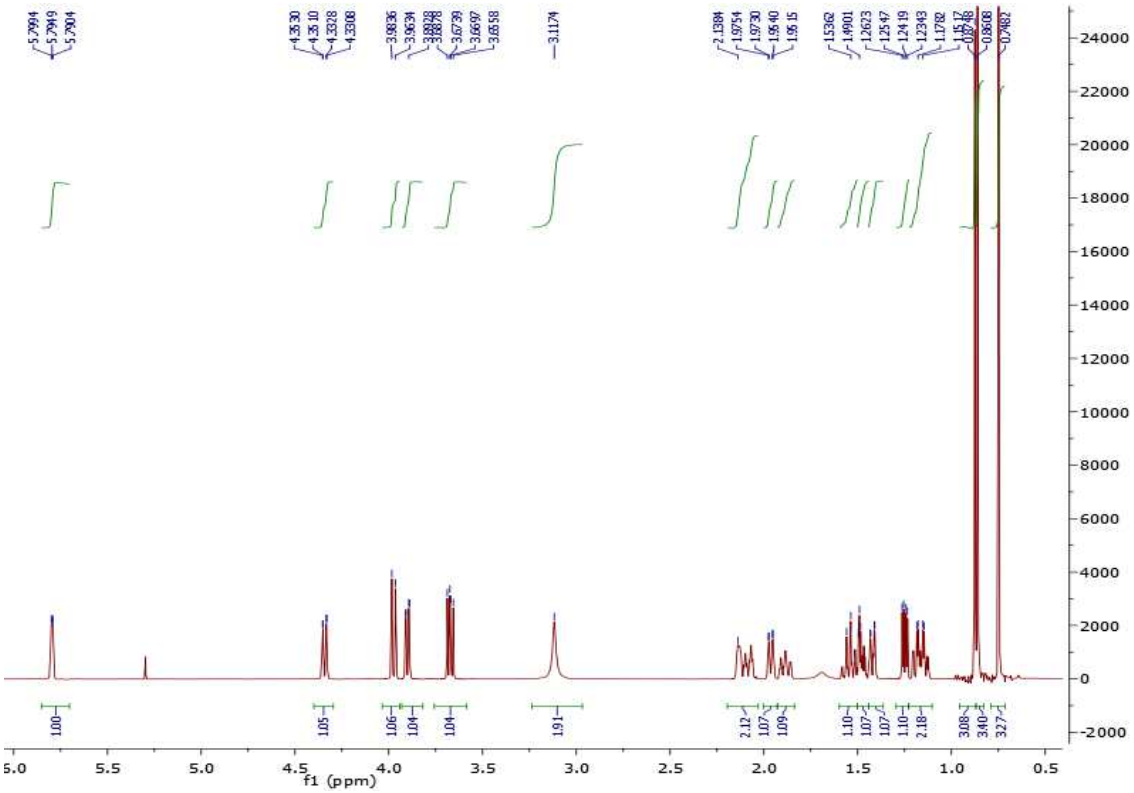


Figure 4. ^{1}H NMR (400 MHz, $CDCl_3$)

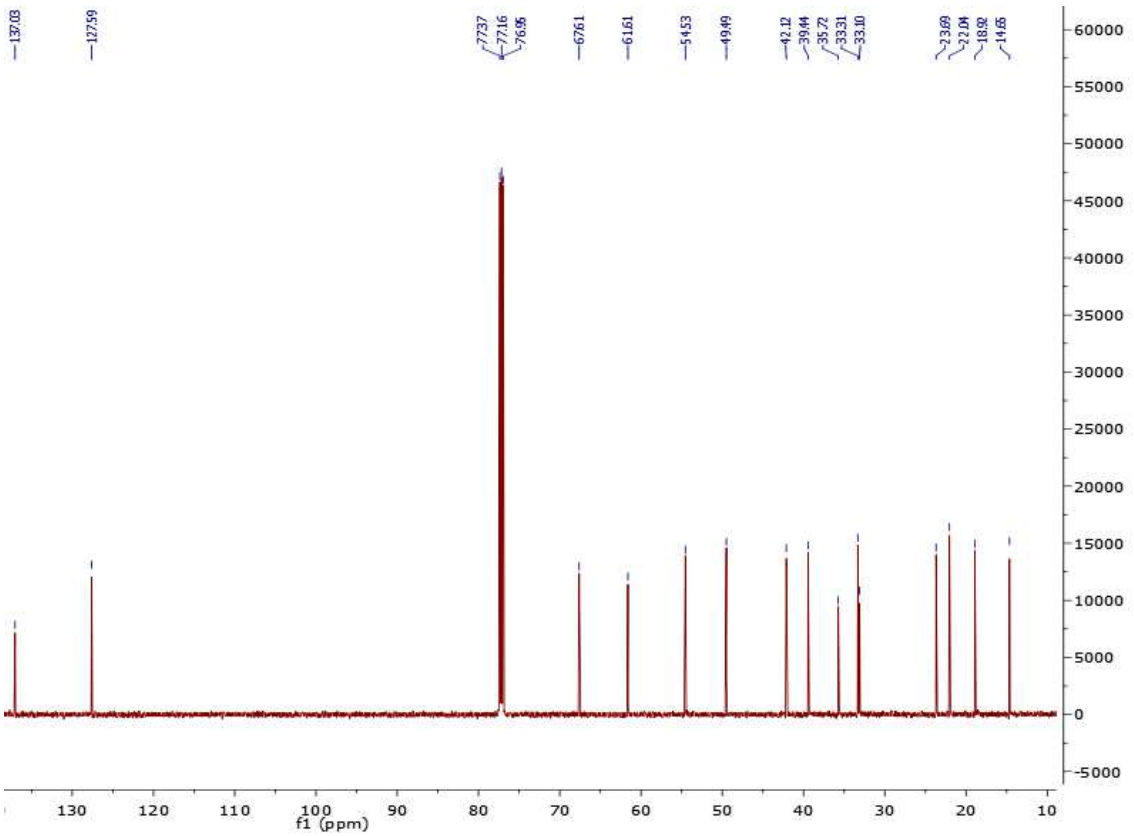


Figure 5. ^{13}C NMR (150 MHz, $CDCl_3$)

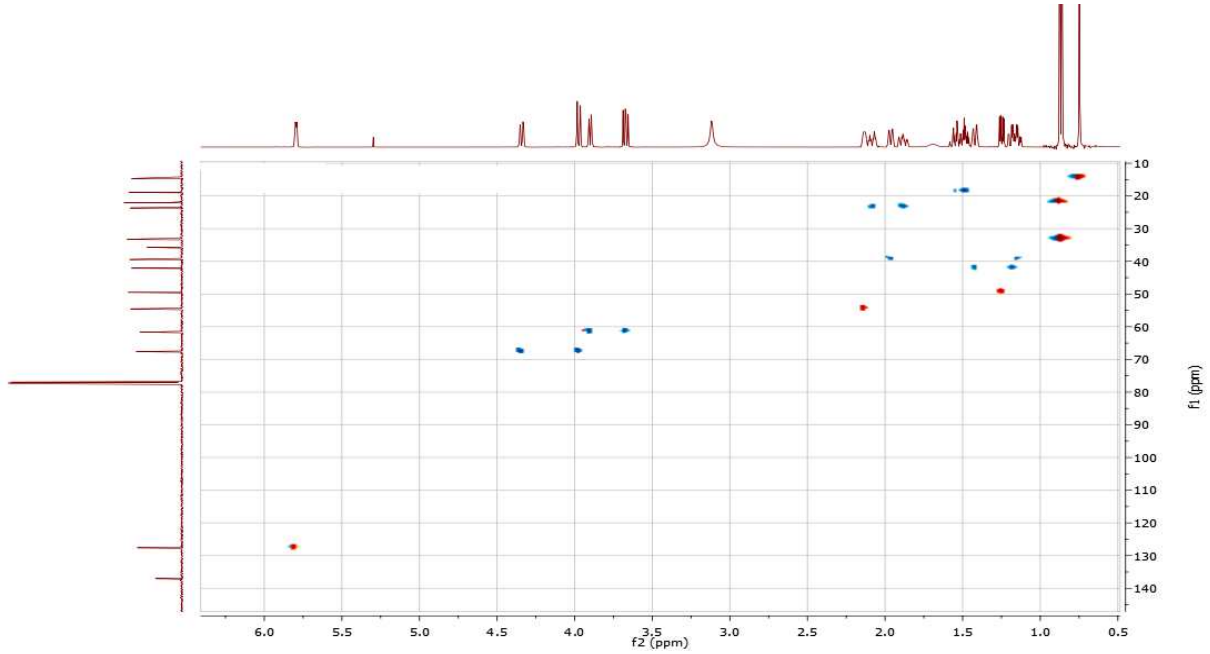


Figure 6. HSQC NMR (400 MHz, $CDCl_3$)

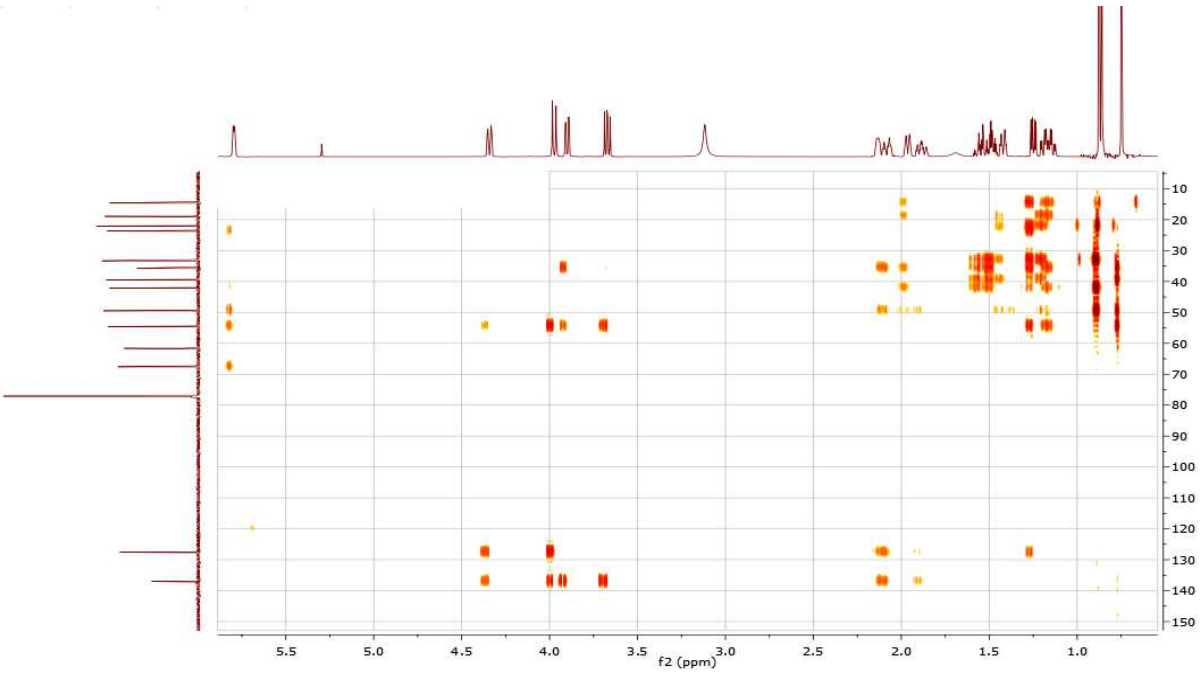


Figure 7. HMBC NMR (400 MHz, $CDCl_3$)

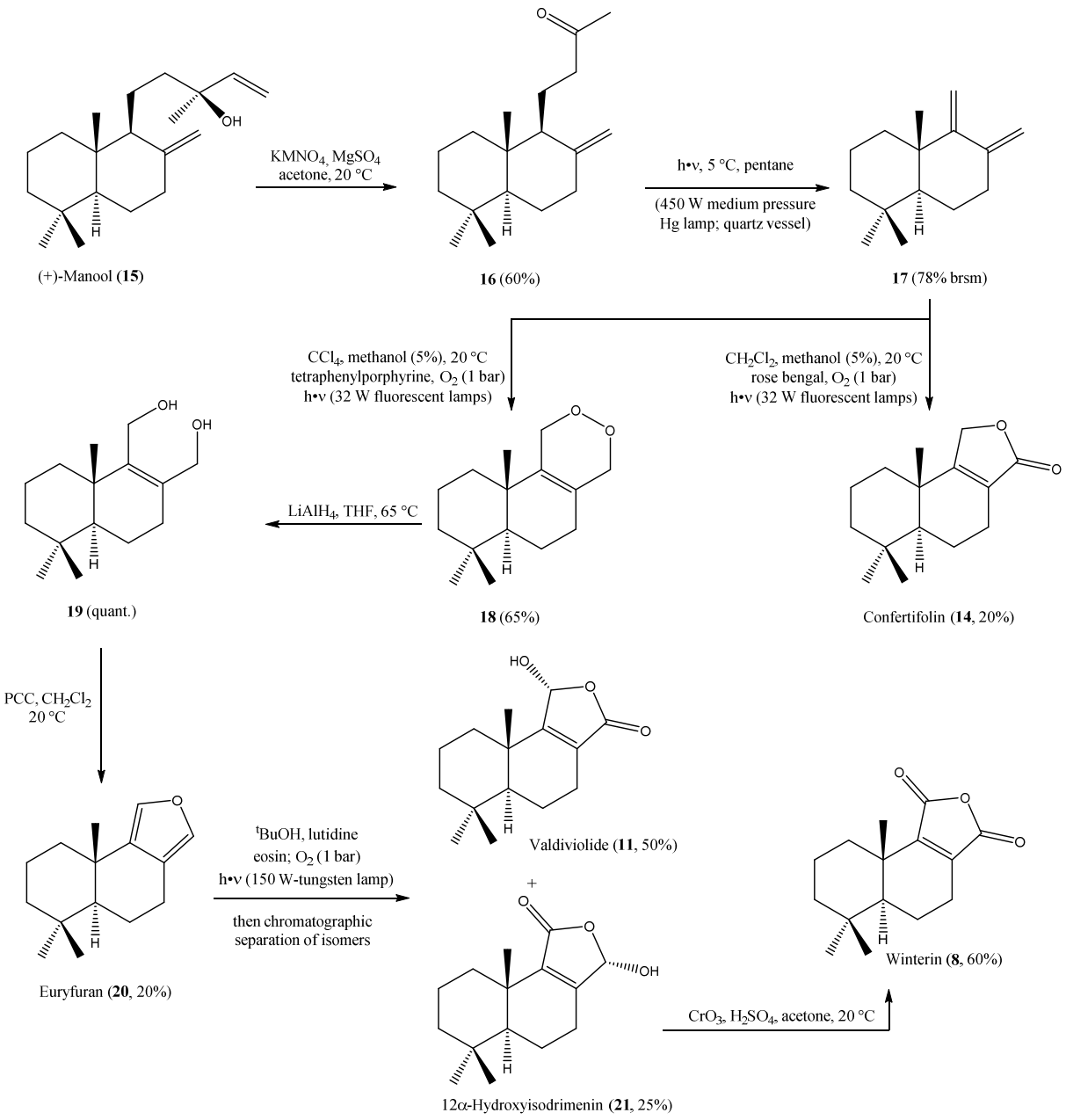
4. CHEMICAL SYNTHESIS OF DRIMANE SESQUITERPENOIDS

The total synthesis of drimanes has been covered in various reviews [6, 20-22]. Therefore, this section is not intended to be comprehensive, but will rather highlight selected examples to illustrate the strategies pursued in the chemical synthesis of drimane sesquiterpenoids.

In general, total syntheses of drimanes can be categorized into “ex-chiral pool” and “de novo” approaches. The former start from other terpenoids, mostly diterpenes, with a decalin moiety already present in the molecular structure, while the latter rely on the construction of the decalin moiety by a synthetic step. Historically, the ex-chiral pool approaches are important because they enabled the first enantioselective semisyntheses of drimane sesquiterpenoids, whereas most de novo syntheses in the 1980’s could only be accomplished in racemic form.

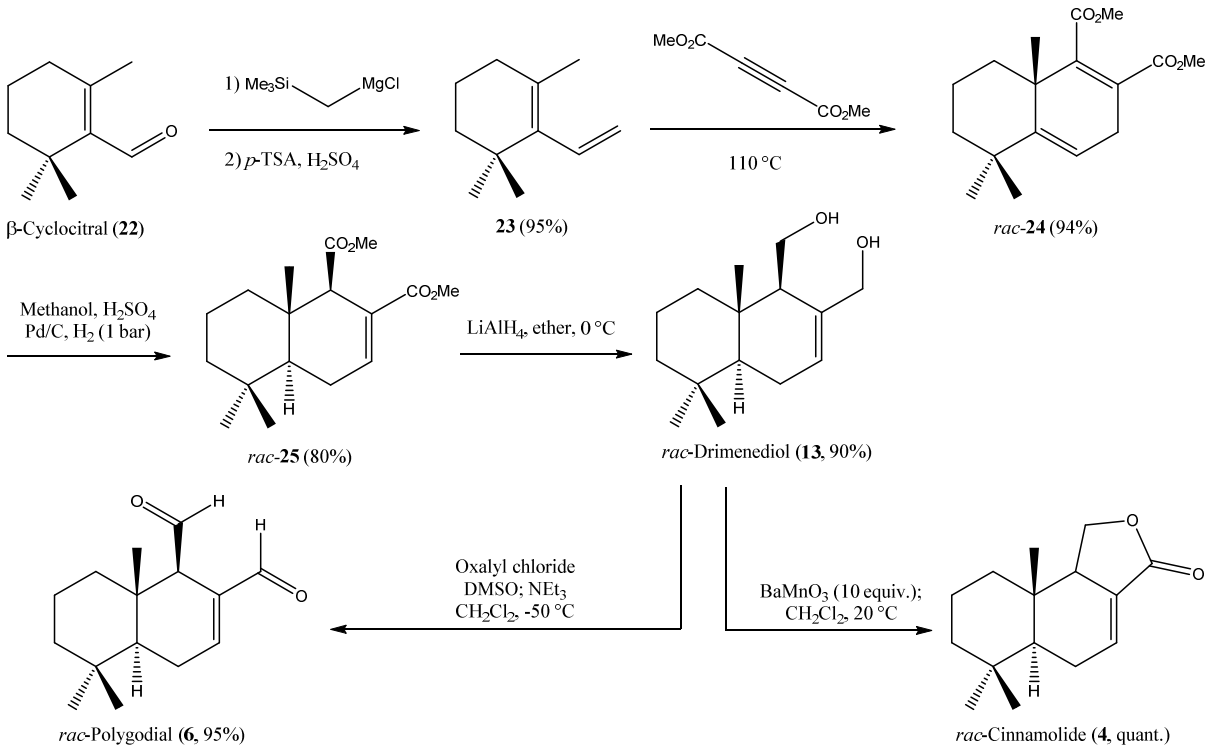
The reviews by Jansen and de Groot [6] and Vlad [20] provide comprehensive overviews of semisyntheses starting from various other terpenoids up to the beginning of the last decade, while the more recent reviews by Salazar and Villamizar focus on the use of the labdane diterpene (+)-manool (**15**) in natural product synthesis [21, 22]. As a representative example, we discuss here semisyntheses of the *Drimys winteri* sesquiterpenoids confertifolin (**14**), valdiviolide (**11**) and winterin (**8**) from (+)-manool (**15**) (Scheme 2). All these syntheses start with a two-step degradation of the side chain at C-9, which proceeds via oxidation to the methyl ketone **16** using permanganate [23] and subsequent Norrish-type-II reaction. This photochemically induced process proceeds with liberation of acetone and formation of diene **17** in 78% yield, based on recovered starting material (brsm) [24]. However, due to the rather low conversion commonly observed for aliphatic Norrish-type II reactions the actual yield of **17** is only 12%. Confertifolin (**14**) was obtained from the key intermediate **17** in low yield by photooxygenation using the dye rose bengal as a photosensitizer. Interestingly, by chosing a different photosensitizer, *meso*-tetraphenyl porphyrin, the cyclic *endo*-peroxide **18** was obtained in good yield [24, 25]. The authors could demonstrate that **18** is not an intermediate *en route* to confertifolin (**14**), but is formed through a different pathway. Endoperoxide **18** turned out to be a useful intermediate in the synthesis of several other drimane sesquiterpenoids. Reductive cleavage of the peroxide with $LiAlH_4$ furnished the 1,4-diol **19** [26], which could be oxidized in one step with pyridinium chlorochromate (PCC) to euryfuran (**20**), a naturally occurring furanosesquiterpene isolated from marine nudibranches or sponges

[27]. Photooxygenation of euryfuran (**20**) furnished a 2:1 mixture of regioisomeric γ-hydroxy lactones, from which the major product was isolated in 50% yield and identified as the *Drimys winteri* sesquiterpene valdiviolide (**11**), whereas the minor isomer was found to be 12α-hydroxyisodrimenin (**21**) [28]. This compound, which is also a natural product isolated from a marine sponge [29], was oxidized with CrO_3 in sulfuric acid to furnish winterin (**8**) [28]. Other, conceptually related approaches to drimanes that involve photochemical or oxidative degradation of the side chain in diterpenoids start from sclareol [30, 31], zamoranic acid [32], labdanolic acid [33], larixol [34, 35] or carnosic acid [36].



Scheme 2. Semisyntheses of representative drimane sesquiterpenes from (+)-manool

As mentioned in the introduction, early "de novo" syntheses of drimanes were racemic. An illustrative example in this regard is the Diels-Alder-route to these sesquiterpenes developed by Ley and coworkers in the early 1980's (Scheme 3) [9].



Scheme 3. "De novo" syntheses of racemic drimanes via Diels-Alder cycloaddition

The conjugated diene **23** required for the construction of the decaline moiety was synthesized from commercially available β-cyclocitral (**22**) by Wittig-olefination or, as shown in scheme 3, by an acid-catalyzed Peterson-olefination. For the Diels-Alder reaction the particularly reactive dienophile acetylene dimethyldicarboxylate was chosen, which reacted with **23** to *rac*-**24** in high yield. Partial hydrogenation with concommitant double bond isomerization required considerable optimization, but was eventually accomplished with Pd/C as a catalyst for the hydrogenation and sulfuric acid to promote the double bond migration. Reduction of the resulting diester *rac*-**25** with $LiAlH_4$ furnished the drimane sesquiterpene *rac*-drimenediol (*rac*-**13**). Ley and coworkers were able to elaborate *rac*-**13** into several other drimane sesquiterpenes, such as cinnamolide (*rac*-**4**), polygodial (*rac*-**6**), isodrimeninol (*rac*-**5**), drimenin (*rac*-**3**) or warburganal, which was not isolated from *Drimys winteri* and is therefore not depicted in Figure 1. As two illustrative examples syntheses of *rac*-cinnamolide

(*rac*-**4**) and *rac*-polygodial (*rac*-**6**) are shown in Scheme 3. Double oxidation of the 1,4-diol *rac*-**13** to the 1,4-dialdehyde polygodial (*rac*-**6**) was accomplished in very high yield using a Swern-oxidation. The synthesis of cinnamolide (*rac*-**4**) required a chemoselective oxidation of the allylic alcohol to the enal, and subsequent oxidation of the resulting lactol to the lactone. For this purpose, the authors tested a variety of methods, such as Collins' reagent ($(pyridine)_2 \bullet CrO_3$), or Fetizon's reagent (Ag_2CO_3 on celite). However, the best choice with regard to yield and cost was barium manganate, which furnished cinnamolide (*rac*-**4**) in quantitative yield. As mentioned above, drimenin has also been synthesized from *rac*-**13**, but its synthesis requires additional protecting group transformations due to the lower reactivity of the primary alcohol at C-11 in oxidation reactions compared to the allylic alcohol at C-12.

The first enantioselective de-novo-approaches to drimane sesquiterpenes made use of either desymmetrization of *meso*-precursors or resolution of racemic precursors. An example for the former is summarized in Scheme 4. Compound **26** (prepared in one step from Hagemanns ester [37]) is first hydrogenated to the *meso*-compound *meso*-**27**, which was efficiently desymmetrized in excellent yield and enantioselectivity, using a lipase from *Pseudomonas fluorescens*, to furnish the enantiopure decaline (-)-**27**. This compound was converted in 16 steps to (+)-**29**, via the intermediate (+)-**28** [38]. This sequence represents a formal total synthesis of drimenin (**3**), because enone (+)-**29** had previously been used to synthesize this particular drimane [39] (Scheme 4).

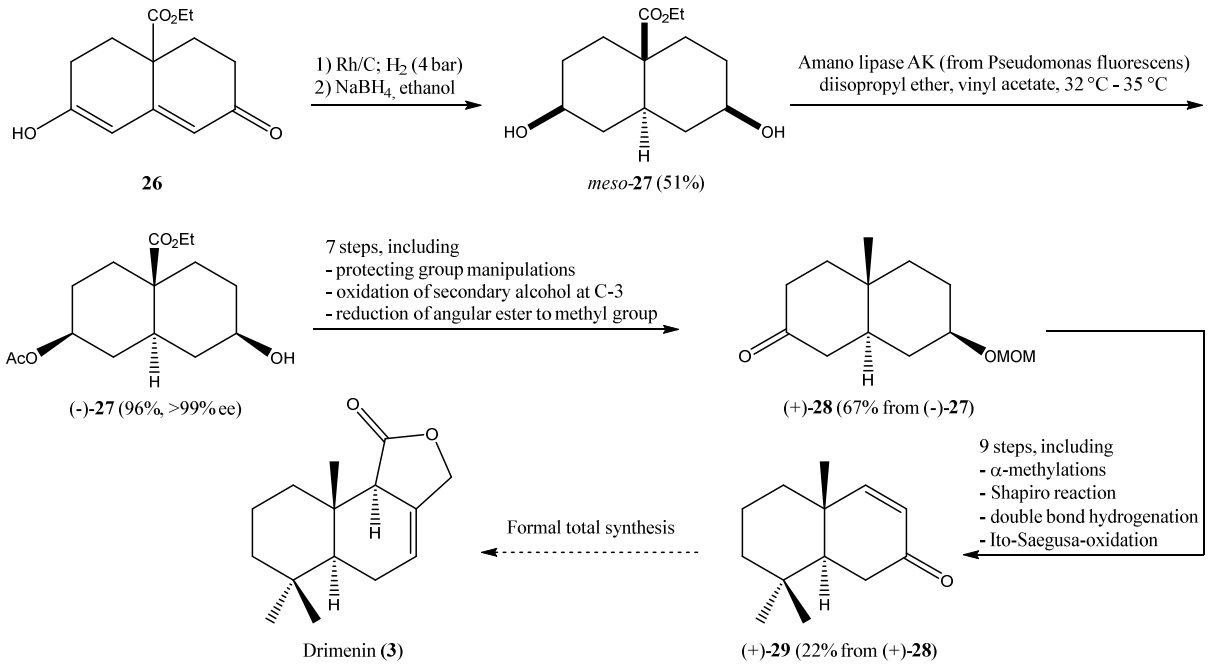
In Scheme 5 two different approaches to the synthesis of enantiopure drimanes or advanced precursors based on optical resolution are highlighted. A useful starting compound is ketone *rac*-**31**, which has been synthesized by Brønsted- [40] or Lewis-acid [41] mediated cyclizations of β-ketoester **30**. Wittig olefination of *rac*-**31** gives an *exo*-methylene compound, the methyl ester of albicanic acid [42]. To set the stage for the optical resolution, the free carboxylic acid (known as albicanic acid) was required. Ester hydrolysis turned out to be troublesome, but could eventually be accomplished using LiI in refluxing DMF.

Optical resolution of *rac*-albicanic acid (*rac*-**32**) was accomplished by the formation of diastereomeric salts with (+)-α-phenylethylamine or (+)-ephedrine, which were separated by crystallization. Subsequent acidification furnished the enantiomers of albicanic acid separately, which were esterified using diazomethane. Through this sequence, (+)- and (-)-methylalbicanate (**31**) could be obtained separately in 50% yield [43]. The authors employed (-)-**31** in the synthesis of several naturally occurring drimanes, e. g. (-)-drimenin ((-)-**3**), which was obtained in two steps from enantiopure **31** by diastereoselective epoxidation to give (+)-

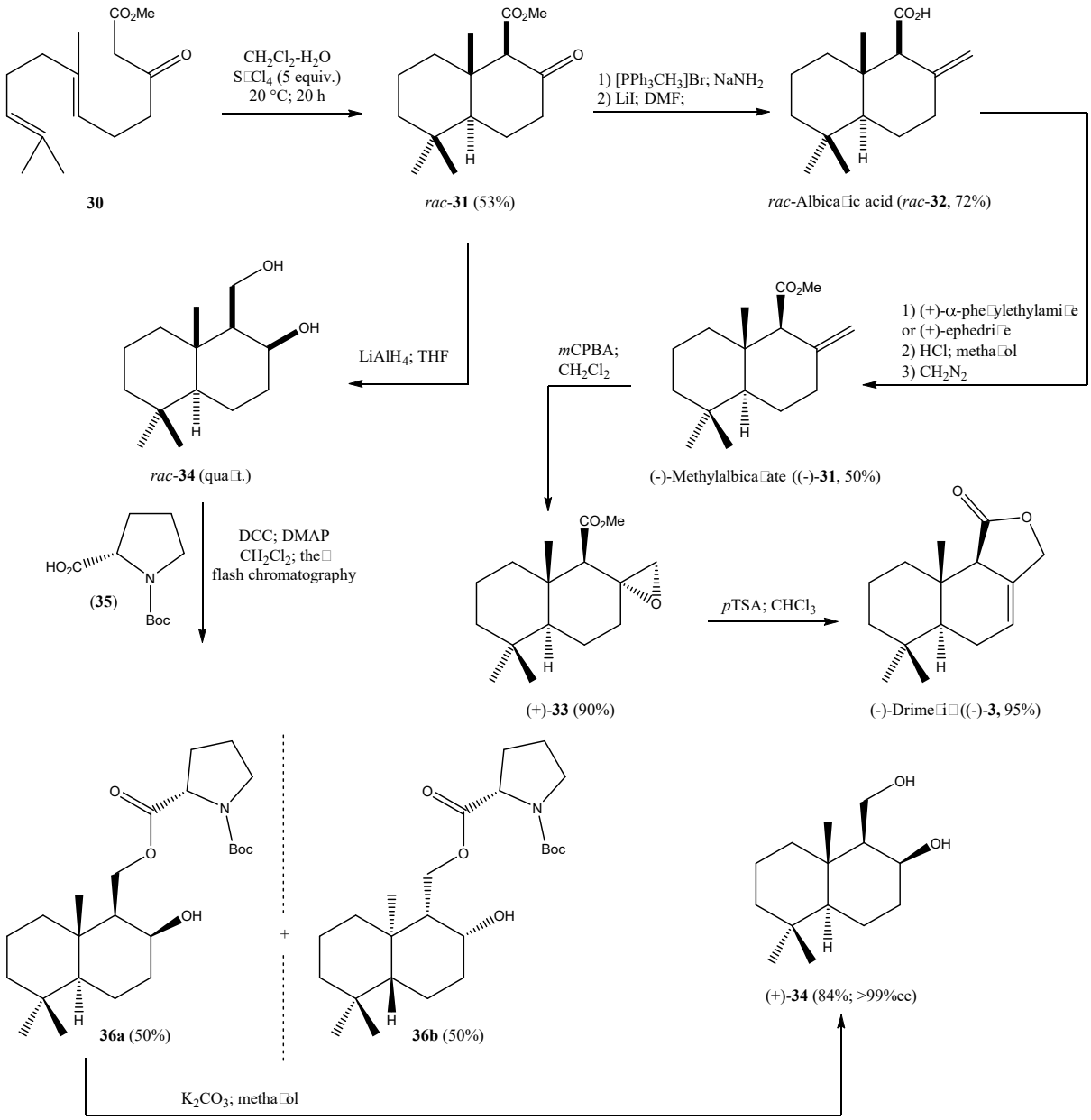
33. This epoxide underwent acid-catalyzed ring opening and elimination of water to give drimenin (Scheme 5) [43].

An alternative method for optical resolutions relies on the formation diastereomers by covalent linkage to a chiral auxiliary. For diol *rac*-**34**, accessible from *rac*-**31** by reduction with $LiAlH_4$, this has been achieved by Steglich esterification of *N*-Boc-proline (**35**) with the primary alcohol of *rac*-**34**. The resulting diastereomers **36a** and **36b** were easily separated by flash chromatography and individually hydrolysed to cleave the proline auxiliary. Both enantiomers of **34** could be obtained in good yield and excellent optical purity, as shown in scheme 5 for (+)-**34** [44, 45].

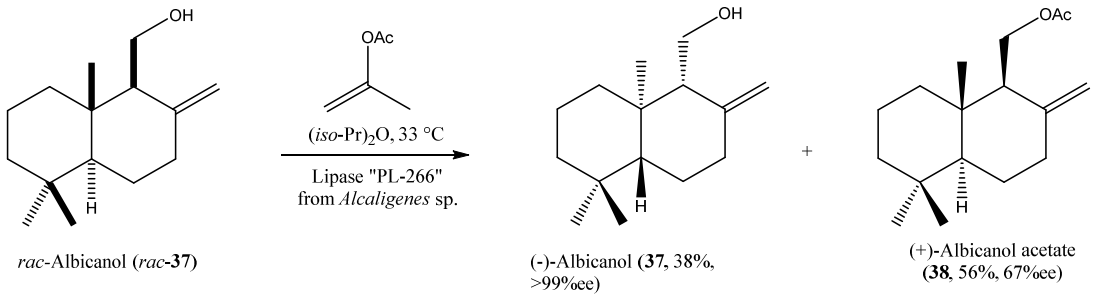
A screening of various enzymes revealed that the lipase PL-266 from *Alcaligenes* sp. furnished enantiomerically pure (-)-albicanol ((-)-**37**) in 38% yield and perfect enantioselectivity with isopropenyl acetate as the acylating reagent. The second product, albicanol acetate (**38**), was obtained in better yield but only with a moderate enantiomeric excess of 67%. Hydrolysis of this enantioenriched (+)-albicanol acetate and re-submission of the resulting enantioenriched albicanol to lipase PL-266 and isopropenyl acetate greatly improved the enantiomeric excess of (+)-albicanol acetate ((+)-**38**) to higher than 99% (**Scheme 6**). Previously, a structurally related ketoalcohol with a primary alcohol at C-11 and a carbonyl group at C-8 had been kinetically resolved using vinyl acetate and a lipase from *Candida cylindracea*, but enantioselectivities remained moderate in this example [47].



Scheme 4. Formal total synthesis of enantiopure drimenin (**3**) through enzymatic desymmetrization

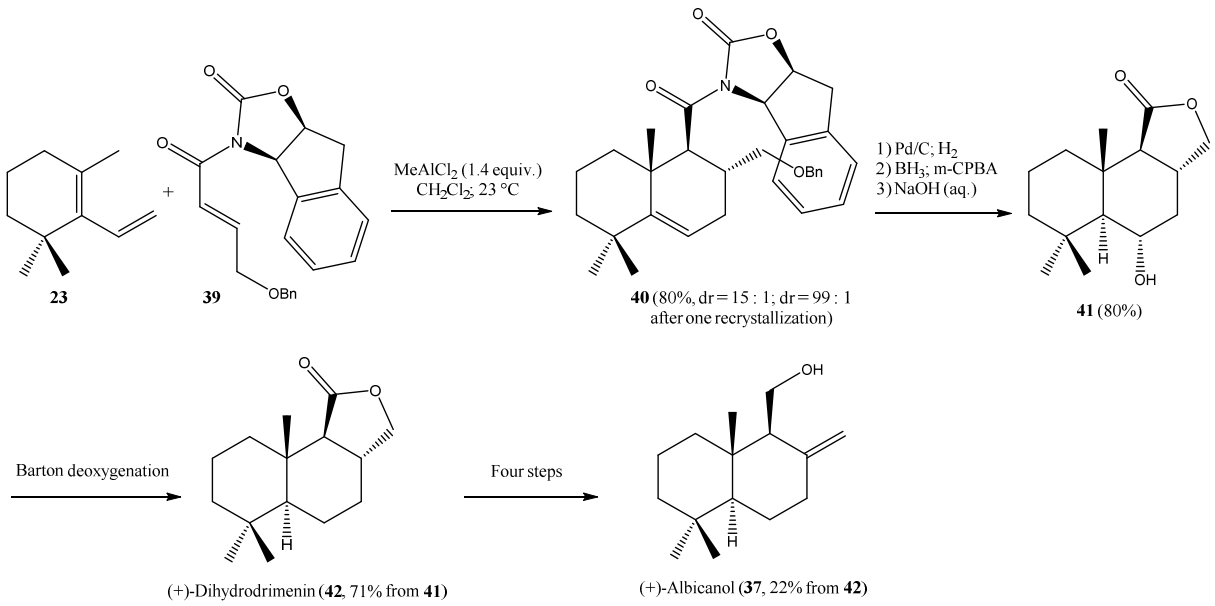


Scheme 5. Optical resolution approaches to enantiopure drimanes



Scheme 6. Enzymatic kinetic resolution of *rac*-albicanol

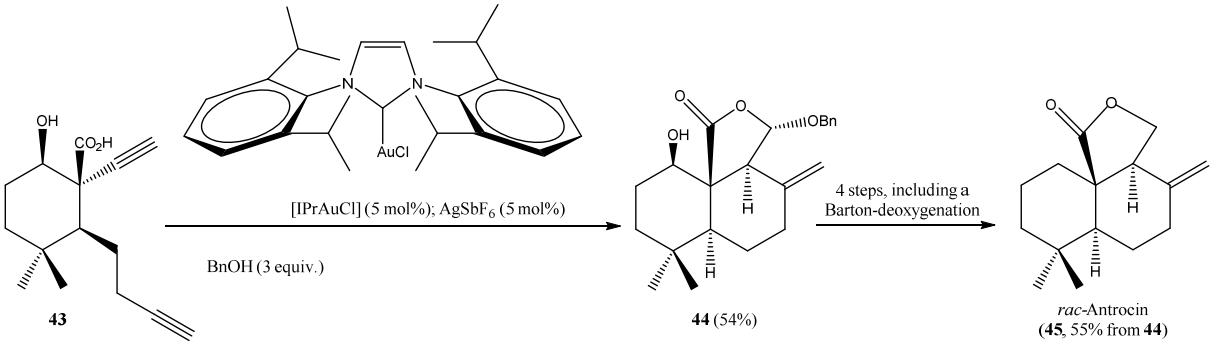
The most recent syntheses of drimane sesquiterpenes use enantioselective methods or transition metal catalysis. An example for the former employs a Diels-Alder reaction of **23** (see Scheme 3) with a chirally modified dienophile **39** for the construction of the decaline ring system [48]. In compound **39** an Evans-auxiliary has been linked to a substituted crotonic acid via an amide bond. A regio- and diastereoselective Diels-Alder reaction was mediated by the Lewis acid $MeAlCl_2$ at ambient temperature, to furnish compound **40** in good yield and as a 15:1 mixture of *exo*- and *endo* isomers. By recrystallization the diastereomeric ratio could be improved to 99:1. The envisaged conversion of the Diels-Alder adduct **40** into the drimane (+)-dihydrodrimenin (**42**) required a detour: originally, the authors investigated the reductive removal of the benzyl protecting group followed by spontaneous lactonization and subsequent hydrogenation of the C-5-C-6 double bond using PtO_2 as a catalyst. These conditions did not result in the formation of dihydrodrimenin, but instead its C-5-epimer was formed in good overall yield. To circumvent this problem the endocyclic double bond was hydroborated to give the drimane **41** with the required configuration at C-5, but with an additional hydroxy group at C-6. Removal of this OH-group was achieved through a Barton deoxygenation, which gave the desired (+)-dihydrodrimenin (**42**) as a single stereoisomer. (+)-Dihydrodrimenin (**42**) could be converted to (+)-albicanol (**37**) in four steps (Scheme 7) [48].



Scheme 7. Enantioselective synthesis of (+)-dihydrodrimenin

Another strategy for the enantioselective construction of the decalin scaffold involves the Lewis acid mediated cyclization of enantiomerically pure polyenes. This approach has not yet been pursued for drimanes from *Drimys winteri*, but for the meroterpenoid (-)-cyclosmenospongine [49], the labdane diterpenoid (-)-andrographolide [50] and the C-14-oxygenated drimane sesequiterpene (+)-iresin [51].

Finally, we highlight one example for the construction of the drimane skeleton via a transition metal catalyzed cascade cyclization of alkynes (Scheme 8) [52]. Diyne **43** was treated with catalytic amounts of the NHC-Au complex [IPrAuCl] and $AgSbF_6$ as a chloride abstractor in the presence of benzylic alcohol as a nucleophile. Electrophilic activation of the C-C-triple bonds induced a cyclization cascade resulting in the advanced intermediate **44**, which was converted in four steps (including the reductive removal of the benzyloxy substituent at C-12 and a Barton-deoxygenation at C-1) to the drimane sesquiterpene antrocin, a secondary metabolite originally isolated from the medicinally used mushroom *Antrodia camphorata*.



Scheme 8. Construction of drimane skeleton via Au-catalyzed cascade cyclization

5. BIOACTIVITY OF DRIMANE

5.1. Effects on ion channels

Drimys winteri has a characteristically pungent flavor in the leaves and barks, which are thick with abundant pungent fat, irritating for eyes, sensible skin or injuries. This is caused by the presence of polygodial (**6**), an unsaturated 1,4-dialdehyde sesquiterpenoid component of the "hot taste" in peppery spices of traditional Japanese cuisine. It adds a pungent

orosensory impression to dishes such as sashimi [53]. In Fig. 1 two drimane sesquiterpenoids with an unsaturated 1,4-dialdehyde at C-8 and C-9 are shown, one of them is polygodial (**6**) and the other is epipolygodial (**7**), the C-9-α-epimer of polygodial. Interestingly, the latter is non-pungent.

The pungency is believed to be based mainly on the activation of two members of the transient receptor potential ankyrin 1 (TRPA1) and transient receptor potential vanilloid 1 (TRPV1). Both are calcium permeable non-selective cation channels which are expressed in the nociceptive neurons and induce a sensation of heat on activation by some pungent ingredients in food. These two polymodic, non-selective cation channels, which also play a role in pain perception, are expressed on free afferent nerve endings of trigeminal neurons in the oral cavity [54]. Capsaicin, isolated from chili peppers, which induces a pungent sensation, was the first agonist reported for TRPV1 [55]. Piperine, one of the key molecules responsible for the pungency of black pepper, also activates TRPV1.

Polygodial increased $[Ca^{2+}]i$ in the CHO cells that expressed hTRPA1, with agonist potency of 0.059 μM and efficacy of 95%, and also demonstrated stronger agonist activity over TRPA1 than TRPV1, suggesting that the pungency of this terpenoid may be due to the activation of TRPA1 [56].

Polygodial also increases the intracellular free calcium concentration $[Ca^{2+}]i$ in human neuroblastoma SH-SY5Y cells, whereas epipolygodial has no effect on the release of $[Ca^{2+}]i$. From this result and the study on inositol phosphate (IP) formation, based on the assumption that there might be a correlation between the release of intracellular Ca^{2+} and pungency of the compounds, it was found that polygodial induces IP mobilization in a concentration dependent way in the SH-SY5Y cells. Phosphoinositide (PPI) turnover was activated by epipolygodial, but only at concentrations 40-fold higher than for polygodial, which emphasizes that not only the 1,4-distance of the aldehydes is important for the biological activity of polygodial, but also the correct configuration [57].

Increasing the intracellular concentration of free calcium ions $[Ca^{2+}]$ by a release from intracellular Ca^{2+} stores, affects directly the noradrenaline (NA) release. For human neuroblastoma cell line SH-SY5Y, preloaded with tritiated NA ($[^3H]$NA), polygodial induced a significant $[^3H]$NA release at concentrations between 0.1 and 0.5 μg/ml with a maximum effect at 0.2 μg/ml. In conclusion, polygodial, at concentrations of 0.1-0.5 μg/ml (equal to 0.4-2 μM), induces NA release which is dependent on polygodial-induced increase in $[Ca^{2+}]$ [58].

Two-pore domain (K2P) potassium channels have been identified as targets for substances associated with typical trigeminal sensations, such as numbing and tingling. The effect of polygodial on human K2P channels TASK-1, TASK-3 was evaluated on *Xenopus oocytes* model. TASK-1 and TASK-3 were both inhibited by polygodial in a dose-dependent manner, with IC_{50} values of 328 ± 24.7 μM for TASK-3 (only partially reversible) and 484.5 ± 18.2 μM for TASK-1 [59].

5.2. Vasorelaxant activities

Vascular diseases, including atherosclerosis, thrombus and vascular inflammation, have become worldwide epidemics in modern society. Vascular diseases affect lumen caliber and induce ischemia, hypoxia and necrosis of tissues and organs, such as acute myocardial ischemia, cerebral infarction and hypertension. To relax or contract blood vessels, vascular endothelial cells (VECs) produce endothelium-derived relaxing factors (EDRFs) and endothelium-derived contracting factors (EDCFs). The balance between EDRFs and EDCFs is essential to maintain vascular tension and endothelial function. In disease status, such as hypertension, abnormal hemodynamic signals disturb the balance between EDRFs and EDCFs, trigger preternatural vasoconstriction and induce endothelial dysfunction.

Polygodial displays vasorelaxant action on the rat portal vein in vitro, which has previously been contracted by various agonists. Polygodial (21-342 μM), preincubated 20 minutes before, produced graded antagonism of the contractile responses caused by bradykinin, endothelin-1, noradrenaline, U46619 (the stable analogue of thromboxane A2), substance P, neurokinin B and senktide (an NK3-selective agonist). At the same concentration, polygodial also produced graded inhibition of the contractile response induced by potassium chloride and by phorbol ester. At the median inhibitory concentration (IC_{50}) level, polygodial was approximately 114- to 177-fold more active in inhibiting mediated contractions to senktide and phorbol ester. When assessed in the tonic contraction induced by endothelin-1 (0.5 nM) or by phorbol (3 μM), polygodial (0.1-100 μM) produced concentration-dependent relaxation, with maximal inhibition (E(max)) of 62 +/- 2% and 100%, respectively. Finally, polygodial (0.1-100 μM) inhibited the rhythmic spontaneous contractions of the rat portal vein (E(max) of 75 +/- 2%). Taken together, these results suggest that the vasorelaxant actions caused by polygodial in rat portal vein are, at least in part, associated with inhibition of calcium influx through voltage-sensitive channels and interaction with protein kinase C-dependent mechanisms [60].

On rings of rabbit pulmonary artery or thoracic aorta and guinea-pig pulmonary artery with endothelium, polygodial induces relaxation. In rings of rabbit or guinea pig pulmonary arteries with and without intact endothelium, polygodial showed EC_{50} values between 0.4 and 2.9 μM. This effect demonstrates the vasorelaxant properties of the compound, which are partly dependent on the release of nitric oxide (NO) from the vascular endothelium and are not related with the opening of K^+ channels [61].

5.3. Antifungal activity

Polygodial exhibits potent fungicidal activity, especially against yeast-like fungi such as *Candida albicans, Cryptococcus neoformans* and *Saccharomyces cerevisiae* [62]. Against *C. albicans*, polygodial displays a minimum inhibitory concentration (MIC) of 3.13 μg/ml and minimum fungicidal concentration (MFC) of 6.25 μg/ml. Under the same conditions miconazole has an MIC of 6.25 μg/ml and an MFC of 50 μg/ml with properties more characteristic offungistaticagents [63, 64]. However, the combination of miconazole with polygodial exhibits strong synergism on both fungistatic and fungicidal action against *C. albicans*. Polygodial completely killed the initial inoculum of *C. albicans* at concentration of 6.25 μg/ml within 12 h, but at a concentration of 3.13 μg/ml no fungicidal activity was observed. Miconazole alone did not exhibit any lethal activity at a concentration of 25 μg/ml within 48 h. However, when miconazole at this concentration mixed with polygodial at a concentration of 3.13 μg/ml was applied, complete lethal action of the mixture was observed within 24 h [65].

Polygodial also shows a synergism on the fungicidal action of anethole, which is an antimicrobial principle from the seeds of *Pimpinella anisum*. The phenylpropanoid anethol has MIC's against *C. albicans* of 200 μg/ml and against *S. cerevisiae* of 200 μg/ml. Polygodial does not enhance the antifungal activity of anethole, but a dramatic increase in the antifungal activity of polygodial occurred when it was combined with a sublethal amount of anethole. The activity of polygodial against *S. cervisiae* and *C. albicans* was increased 64- and 32-fold, respectively. In other words, the MIC of polygodial against *S. cervisiae* was reduced from 1.56 to 0.024 μg/ml, and in the case of *C. albicans* from 3.13 to 0.098 μg/ml, when polygodial was combined with 100 μg/ml of anethole (which corresponds to half of the MIC of anethole against both *C. albicans* and *S. cervisiae*) [66].

The fungicidal activity of polygodial has multiple origins, but it is primarily caused by its ability to act as a nonionic surface-active agent disrupting the lipid-protein interface [67] and thereby inducing membrane leakage in *S.cerevisiae* and also in the human neuroblastoma cells [68]. Due to the 1,4-dialdehyde structure, polygodial can react with primary amines to pyrroles. Considering that lipids such as phosphatidylethanolamine (PE) and phosphatidylserine (PS) contain a primary amino group, polygodial mayreact with the amino groups of PE and PS at the outer leaflet of plasma membranes, thereby disturbing the balance of the plasma membrane. Once polygodial enters the cytoplasm by destroying the membrane barrier, it is involved in biochemical processes, e.g. by reacting with l-cystein-containing cytoplasmic materials, such as the small moleculeglutathione, and the proteinalcohol dehydrogenase, to potentiate the antifungal action [69].

5.4. Antibacterial activity

Drimane sesquiterpenoids are not potent antibacterial compounds. For instance, drimenol has antibacterial activities (MIC values) against *S. aureus* of 667 μg/ml, *B. cereus* of 667 μg/ml, *A. baumanii* of 583 μg/ml, *E. coli* of 1.333 μg/ml and *P. aeruginosa* of 667 μg/ml [70]. These MIC values are more than 100 times higher than the corresponding values for polygodial, which is the most active metabolite with regard to antibacterial activity.

Polygodial displays minimum bactericidal concentrations (MBC) against Gram-positive bacteria *Bacillus subtilis* of 100 μg/ml (0.34 mM) and *Staphylococcus aureus* of 100 μg/ml (0.34 mM). Against Gram-negative bacteria the following MBC values were reported: *Escherichia coli* of 100 μg/ml; *Salmonella choleraesuis* of 50 μg/ml. Polygodial did not display activity up to 400 μg/ml against *Pseudomonas aeruginosa* strains [71].

Drimanes also affect the bacterial quorum sensing system (QS), which is the communication system through which bacteria can regulate colonization, biofilm formation and virulence factors. The evaluation of polygodial, drimenol, isodrimeninol, drimenin, isodrimenin, cinnamolide and valdiviolide in the bacterial communication system through the biosensor AI-1, *Chromobacterium violaceum* ATCC 12472 show that cinnamolide and valdiviolide, two α,β unsaturated lactones with the carbonyl on position 12 of the drimane skeleton are inhibitors of QS, displaying a reduction of the pigment violacein at 54.3% and 43.7%, respectively, using a concentration of sesquiterpene of 800 μg/ml [11].

5.5. Insecticidal activity of drimane sesquiterpenoids

Antifeedant activity and toxicity of drimane sesquiterpenes have been investigated in the search for new natural and non-toxic compounds for pest control. In spite of all efforts in protecting crops from noxious pests all over the world, losses due to these causes can annually reach 10-20% [72].

Drimendiol, isodrimeninol, isotadeonal and polygodial were investigated against the Egyptian cotton leafworm, *Spodoptera littoralis* (Boisduval) (*Lep. Noctuidae*). At 1000 ppm, polygodial exhibited the strongest antifeedant activity against sixth instar larvae when applied on leaf discs under choice and no-choice conditions (feeding deterrence index: 94.7% for polygodial, on the choice test). Nutritional indices after exposure over a 12 h period show reduced feeding and growth rates, being significantly higher with polygodial and isodrimeninol. Polygodial was the most potent feeding and growth inhibitor for *S. littoralis* with DC_{50} = 708 ppm and EC_{50} = 198 ppm, respectively [73].

Polygodial shows antifeedant and deterrent effectsagainst a broad spectrum of insects. For example, its ED_{50} (effective dose at which 50% of the insects are deterred) value for the silverleaf whitefly *Bemisia tabaci* (Gennadius) is about 25 μg/g fresh weight (FW), and the ED50 value for the green peach aphid *M. persicae* is about 54 μg/g FW. Bioassays were benchmarked with pyrethrins that had a 20-fold lower ED_{50} of approximately 1.4 μg/g FW against whiteflies, but only a twofold lower ED_{50} (about 28 μg/g FW) against peach aphids. Polygodial also showed moderate phytotoxic effects (score of 2 on a scale of 1-5) on tomato leaves at concentrations above the ED_{50} (≥90 μg/g FW) [74].

A. aegypti is a vector of arboviruses responsible for major diseases like dengue and dengue hemorrhagic fever, West Nile Virus and chikungunya. It infects more than 100 million people every year. Confertifolin displays LC_{50} values of 2.90 and 2.96 ppm for second and fourth instar larvae of *A. aegypti*, respectively. At 10 ppm, this concentration of confertifolin showed ovicidal activity of 100, 100, and 77.6% on 0-6, 6-12, and 12-18 hold eggs, respectively. The repellent activity was 323.2 min, oviposition deterrent activity was 97.52% and adulticidal activity was 100% against *A. aegypti*. Confertifolin shows statistically significant activity against *A. aegypti* and might be considered as a potent source for the production of superior natural mosquitocides [75].

Leishmaniasis and American trypanosomiasis (Chagas' disease) are tropical diseases caused by protozoan parasites belonging to the genera *Leishmania* and *Trypanosoma* which affect the poorest population in developing countries. The clinically used drugs include highly

toxic compounds such as pentavalent antimonials and nitroheterocyclic compounds. Polygodial shows anti-leishmanial and anti-trypanosomal activities against all tested *Leishmania* species. IC_{50} values are in the range between 34.83 and 62.11 μg/ml, with high parasite selectivity towards *Trypanosoma cruzi* trypomastigotes (2 μg/ml). Polygodial also showed intense ultrastructural damages in *Leishmania* in short-time incubation. Despite its effectiveness, polygodial was not more active than the standard drug pentamidine, which has IC_{50} values between 0.23 and 0.39 μg/ml [76].

SOLUTION OF PROBLEM 1: DRIMENDIOL (**13**).

ACKNOWLEDGEMENTS

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REFERENCES

1. Forster JR, Forster JGA. Characteres Generum Plantarum. London, England: 1775, 84, t. 42.
2. Wilhelm Mösbach E. Botánica indígena de Chile. Santiago, Chile: Editorial Andrés Bello; 1992. pp. 78-79.
3. Appel HH. Estudio quimico de la corteza del árbol *Drimys winteri* Forst. Scientia. 1948; 15: 31-32.
4. Appel HH, Brooks CJW, Overton KH. The constitution and stereochemistry of drimenol, a novel bicyclic sesquiterpenoid. J Chem Soc. 1959; 33: 32.
5. Appel HH, Bond RPM, Overton KH. Sesquiterpenoids - III: The constitution and stereochemistry of valdiviolide, fuegin, winterin and futronolide. Tetrahedron. 1963; 19: 635-641.
6. Jansen BJ, de Groot AE. Occurrence, biological activity and synthesis of drimane sesquiterpenoids. Nat Prod Rep. 2004; 21: 449-477.
7. Bastos JK, Kaplan MAC, Gottlieb OR. Drimane-type sesquiterpenoids as chemosystematic markers of Canellaceae. J Braz Chem Soc. 1999; 10: 136-139.
8. Toyota M, Ooiso Y, Kusuyama T, Asakawa Y. Drimane-type sesquiterpenoids from the liverwort *Diplophyllum serrulatum*. Phytochemistry. 1994; 35: 1263-1265.

9. Hollinshead DM, Howell SC, Ley SV, Mahon M, Ratcliffe NM, Worthington PA. The Diels-Alder route to drimane related sesquiterpenes; synthesis of cinnamolide, polygodial, isodrimeninol, drimenin and warburganal. J Chem Soc Perkin Trans. 1983; 1: 1579-1589.
10. Maurs M, Azerad R, Cortes M, Aranda G, Bertrann Delahaye M, Ricard L. Microbial hydroxylation of natural drimenic lactones. Phytochemistry. 1999; 52: 291-296.
11. Carcamo G, Silva M, Becerra J, Urrutia H, Sossa K, Paz C. Inhibition of quorum sensingby drimane lactones from Chilean flora. J Chil Chem Soc. 2014; 59: 2622.
12. Brito I, López-Rodríguez M, Zárraga M, Paz C, Pérez C. 6,6,9a-trimethyl-5,5a,6,7,8,9,9a,9b-octahydronaphtho[1,2-c]furan-1(3h)-one. Acta Cryst. 2008; 64: 738.
13. Brito I, López-Rodríguez M, Zárraga M, Paz C, Pérez C. A cinnamolide-class sesquiterpene lactone from *Drimys winteri* forst. var. Chilensis. J Chil Chem Soc. 2008: 59; 1732-1733.
14. Paz C, BurgosV, Suarez S, Baggio R. A monoclinic form of dendocarbin A: a borderline case of one-dimensional isostructural polymorphism. Acta Cryst. 2015; 71: 294-297.
15. Paz C, Mercado D, Suarez S, Baggio R. Two drimane lactones, valdiviolide and 11-epivaldiviolide, in the form of a 1:1 cocrystal obtained from *Drimys winteri* extracts. Acta Cryst. 2014; 70: 1112-1115.
16. Ayer WA, Trifonow LS. Drimane sesquiterpene lactones from *Peniophora polygonia.* J Nat Prod. 1992; 55: 1454-1461.
17. Gaspar H, Gavagnin M, Calado G, Castelluccio F, Mollo E, Cimino G. Pelseneeriol-1 and -2: new furanosesquiterpene alcohols from porostome nudibranch *Doriopsilla pelseneeri*. Tetrahedron. 2005; 61: 11032-11037.
18. Rodriguez B, Zapata N, Medina P, Viñuela E. A complete ^{1}H and ^{13}C NMR data assignment for four drimane sesquiterpenoids isolated from *Drimys winterii.* Magn Reson Chem. 2005; 43: 82-84.
19. Rukachaisirikul V, Khamthong N, Sukpondma Y, Phongpaichit S, Hutadilok-Towatana N, Sakayaroj Graidist PJ, Kirtikara K. Cyclohexene, diketopiperazine, lactone and phenol derivatives from the sea fan-derived fungi *Nigrospora* sp. PSU-F11 and PSU-F12. Arch Pharm Res. 2010; 33: 375-380.
20. Vlad PF. Synthetic investigations in the field of drimane sesquiterpenoids, in studies in natural products chemistry. Rahman, A.-U., Ed.Elsevier; 2006. pp. 393-432.
21. Salazar FJ, Villamizar JE. Use of (+)-manool in the synthesis of natural products. Part II. Diterpenes and relatives. J Chem Res. 2013; (S): 63-70.
22. Salazar FJ, Villamizar JE. Use of (+)-manool in the synthesis of natural products. Part I. Sesquiterpenes. J Chem Res. 2013: 1-5.

23. Do Khac Manh D, Fetizon M, Flament JP. Synthese de diterpenes tetracycliques du type hibane. Tetrahedron. 1975; 31: 1897-1902.

24. Nakano T, Maillo MA. A novel, short synthesis of confertifolin. Synth Commun. 1981; 11: 463-473.

25. Ciocarlan A, Edu C, Biriiac A, Lungu L, Aricu A, D'Ambrosio M, Shova S, Nicolescu A, Deleanu C, Vornicu N. Synthesis of polyfunctional drimanes from drim-7,9(11)-diene and drim-8-en-7-one. Synth Commun. 2013; 43: 3020-3033.

26. Nakano T, Martin A. A very short, highly efficient synthesis of natural (-)-warburganal. J Chem Res. 1989; (S): 52-53.

27. Nakano T, Maillo MA, Rojas A. Reaction of (1S,6S)-1,7,7-trimethyl-2,3-dimethylene-trans-bicyclo[4.4.0]decane with thallium(III) acetate; a new route to (-)-warburganal and (+)-euryfuran. J Chem Soc Perkin Trans. 1987; 1: 2137-2140.

28. Nakano T, Villamizar J, Maillo RA. Synthesis of drimanic sesquiterpenes, (+)-valdiviolide, (+)-12 alpha-hyroxyisodrimenin and (+)-winterin. J Chem Res. 1998; (S): 560-561.

29. Montagnac A, Martin MT, Debitus C, Païs M. Drimane sesquiterpenes from the sponge *Dysidea fusca*. J Nat Prod. 1996; 59: 866-868.

30. Barrero AF, Manzaneda EA, Altarejos J, Salido S, Ramos JM, Simmonds MSJ, Blaney WM. Synthesis of biologically active drimanes and homodrimanes from (-)-sclareol. Tetrahedron. 1995; 51: 7435-7450.

31. Barrero AF, Cortés M, Manzaneda EA, Cabrera E, Chahboun R, Lara M, Rivas AR. Synthesis of 11,12-epoxydrim-8,12-en-11-ol, 11,12-diacetoxydrimane, and warburganal from (−)-sclareol. J Nat Prod. 1999; 62: 1488-1491.

32. Urones JG, Marcos IS, Perez BG, Lithgow AM, Diez D, Basabe P, Gomez PM. Transformation of labdanes into drimanes. Semisyntheses of poligodial and warburganal from zamoranic acid. Nat Prod Lett. 1994; 5: 21-24.

33. Lithgow AM, Marcos IS, Basabe P, Sexmero MJ, Díez D, Gómez A, Estrella A, Urones JG. Labdanolic acid as synthetic precursor of active drimanes. Nat Prod Lett. 1995; 6: 291-294.

34. Vlad PF, Ciocarlan A, Coltsa M, Edu C, Biriiac A, Barba A, Deleanu C, Nicolescu A, D'Ambrosio M, de Groot A. Synthesis of (−)-albrassitriol and (−)-6-epi-albrassitriol from (+)-larixol. Nat Prod Res. 2013; 27: 809-817.

35. Vlad PF, Ciocarlan A, Edu C, Aricu A, Biriiac A, Coltsa M, D'Ambrosio M, Deleanu C, Nicolescu A, Shova S, Vornicu N, de Groot, A. Regio- and stereoselective synthesis of (+)-6-ketoeuryfuran, (+)-6-ketowinterin, and (−)-7-ketoeuryfuran from accessible labdane diterpenoids (+)-larixol and (−)-sclareol. Tetrahedron. 2013; 69: 918-926.

36. Li FZ, Li S, Zhang PP, Huang ZH, Zhang WB, Gong J, Yang Z. A chiral pool approach for asymmetric syntheses of (-)-antrocin, (+)-asperolide C, and (-)-trans-ozic acid. Chem Commun. 2016; 52: 12426-12429.
37. Hussain S, Brotherhood PR, Judd LW, Davis AP. Diaxial diureido decalins as compact, efficient, and tunable anion transporters. J Am Chem Soc. 2011; 133: 1614-1617.
38. Toyooka N, Nishino A, Momose T. Enantiodivergent synthesis of a decalin type of chiral building blocks and their application to terpenoid synthesis. Tetrahedron. 1997; 53: 6313-6326.
39. Wenkert E, Strike DP. Synthesis of some drimanic sesquiterpenes. J Am Chem Soc. 1964; 86: 2044-2050.
40. Stadler PA, Nechvatal A, Frey AJ, Eschenmoser A. Untersuchungen über den sterischen Verlauf säure-katalysierter Cyclisationen bei terpenoiden Polyenverbindungen. 1. Mitteilung. Cyclisation der 7,11-Dimethyl-2(trans), 6(trans), 10-dodecatrien-und der 7, 11-Dimethyl-2(cis), 6(trans), 10-dodecatrien-säure. Helv Chim Acta. 1957; 40: 1373-1409.
41. White JD, Skeean RW, Trammell GL. Lewis acid and photochemically mediated cyclization of olefinic beta-keto esters. J Org Chem. 1985; 50: 1939-1948.
42. Liapis M, Ragoussis V, Ragoussis N. Improved total synthesis of (+/-)-drimenin. J Chem Soc Perkin Trans. 1985; 1: 815-817.
43. Ragoussis V, Liapis M, Ragoussis N. Synthesis and resolution of albicanic acid. Simple access to optically active drimane sesquiterpenes. J Chem Soc Perkin Trans. 1987; 1: 987-992.
44. Toshima H, Oikawa H, Toyomasu T, Sassa T. Total synthesis of both enantiomers of copalol via optical resolution of a general synthetic intermediate for drimane sesquiterpenes and labdane diterpenes. Tetrahedron. 2000; 56: 8443-8450.
45. Toshima H, Oikawa H, Toyomasu T, Sassa T. Total synthesis of (+)-albicanol and (+)-albicanyl acetate. Biosci Biotechnol Biochem. 2001; 65: 1244-1247.
46. Akita H, Nozawa M, Mitsuda A, Ohsawa H. A convenient synthesis of (+)-albicanol based on enzymatic function: total syntheses of (+)-albicanyl acetate, (−)-albicanyl 3,4-dihydroxycinnamate, (−)-drimenol, (−)-drimenin and (−)-ambrox. Tetrahedron: Asymmetry. 2000; 11: 1375-1388.
47. Nair MS, Anilkumar AT. Versatile chiral intermediates for terpenoid synthesis using lipase catalysed acylation. Tetrahedron: Asymmetry. 1996; 7: 511-514.
48. Henderson JR, Parvez M, Keay BA. A concise Diels-Alder strategy for the asymmetric synthesis of (+)-albicanol, (+)-albicanyl acetate, (+)-dihydrodrimenin, and (−)-dihydroisodrimeninol. Org Lett. 2009; 11: 3178-3181.

49. Speck K, Magauer T. Evolution of a polyene cyclization cascade for the total synthesis of (−)-cyclosmenospongine. Chem Eur J. 2017; 23: 1157-1165.
50. Gao HT, Wang BL, Li WDZ.Synthetic applications of homoiodo allylsilane II. Total syntheses of (−)-andrographolide and (+)-rostratone. Tetrahedron. 2014; 70: 9436-9448.
51. Wang BL, Gao HT, Li WDZ. Total synthesis of (+)-iresin. J Org Chem. 2015; 80: 5296-5301.
52. Shi H, Fang L, Tan C, Shi L, Zhang W, Li Cc, Luo T, Yang Z. Total syntheses of drimane-type sesquiterpenoids enabled by a gold-catalyzed tandem reaction. J Am Chem Soc. 2011; 133: 14944-14947.
53. Ohsuka A. The structure of tadeonal and isotadeonal components of the *Polygonum hydropiper* L. Nippon Kagaku Zasshi. 1963; 84: 748-752.
54. Calixto JB, Kassuya CA, André E, Ferreira J. Contribution of natural products to the discovery of thr transient reporter potential (TRP) channels family and their functions. Pharmacol. Ther. 2005: 106; 179-208.
55. Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature. 1997: 23; 816-824.
56. Iwasaki Y, Tanabe M, Kayama Y, Abe M, Kashio M, Koizumi K, Okumura Y, Morimitsu Y, Tominaga M, Ozawa Y, Watanabe T. Miogadial and miogatrial with α,β-unsaturated 1,4-dialdehyde moieties - novel and potent TRPA1 agonists. Life Sci. 2009; 85: 60-69.
57. Forsby A, Walum E. Polygodial induces inositol phosphate turnover in human neuroblastoma SH-SY5Y cells. Neurosci Lett. 1996; 217: 50-54.
58. Andres MI, Forsby A, Walum E. Polygodial-induced noradrenaline release in human neuroblastoma SH-SY5Y cells. Toxicol in Vitro. 1997; 11: 509-511.
59. Beltrán LR, Dawid C, Beltrán M, Gisselmann G, Degenhardt K, Mathie K, Hofmann T, Hatt H. The pungent substances piperine, capsaicin, 6-gingerol and polygodial inhibit the human two-pore domain potassium channels TASK-1, TASK-3 and TRESK. Front Pharmacol. 2013; 4: 141.
60. El Sayah M, Filho VC, Yunes RA, Malheiros A, Calixto JB. Action of polygodial on agonist-induced contractions of the rat portal vein in vitro. J Cardiovasc Pharmacol. 2000; 35: 670-675.
61. André E, Malheiros A, Cechinel-Filho V, Yunes RA, Calixto JB. Mechanisms underlying the relaxation caused by the sesquiterpene polygodial in vessels from rabbit and guinea-pig. Eur J Pharmacol. 1999; 386: 47-53.

62. LeeSH, LeeJR, Lunde CS, Kubo I. *In vitro* anti-fungal susceptibilities of *Candida albicans* and other fungal pathogens to polygodial, a sesquiterpene dialdehyde. Planta Medica. 1999; 65: 204-208.
63. Fromtling RA. Overview of medically important antifungal azole derivatives. Clin Microbiol Rev. 1988; 1: 187-217.
64. Como JA, DismukesWE. Oral azole drugs as systemic antifungal therapy. New Engl J Med. 1994; 330: 263-272.
65. Kubo I, Lee SH, Shimizu K. Combination effect of miconazole with polygodial against *Candida albicans*. Open J Med Microbiol. 2011; 1: 7-11.
66. Kubo I, Himejima M. Anethole, a synergist of polygodial against filamentous microorganisms. J Agric Food Chem. 1991; 39: 2290-2292.
67. Kubo I, Fujita K, Lee SH. Antifungal mechanism of polygodial. J Agric Food Chem. 2001; 49: 1607-1611.
68. Forsby A, Walum E, Sterner O. The effect of six sesquiterpenoids unsaturated dialdehydes on cell membrane permeability in human neuroblastoma SH-SY5Y cells. Chem-Biol Interact. 1992; 84: 85-95.
69. Fujita K, Kubo I. Multifunctional action of antifungal polygodial against *Saccharomyces cerevisiae*: Involvement of pyrrole formation on cell surface in antifungal action. Bioorg Med Chem. 2005; 13: 6742-6747.
70. Santos TG, Iêda JD, Begnini M, Rebelo RA, Verdi M, de Gasper AL, Dalmarco EM. Chemical characterization of essential oils from *Drimys angustifolia* Miers (Winteraceae) and antibacterial activity of their major compounds. J Braz Chem Soc. 2013; 24: 164-170.
71. Kubo I, Fujita K, Lee SH, Ha TJ. Antibacterial activity of polygodial. Phytother Res. 2005; 19: 1013-1017.
72. Fornal J, Jelinski T, Sadowska J, Grunda S, Nawrot J, Niewiada A, Warchalenski JR, Blaszczak W. Detection of granary weevil *Sitophilus granarius* (L.) eggs and internal stage analysis. J Stored Prod Res. 2007; 43: 142-148.
73. Zapata N, Budia F, Vinuela E, Medina P. Antifeedant and growth inhibitory effects of extracts and drimanes of *Drimys winteri* stem bark against *Spodoptera littoralis* (Lep., Noctuidae). Indust Crops Prod. 2009; 30: 119-125.
74. Prota N, Bouwmeester HJ, Jongsma MA. Comparative antifeedant activities of polygodial and pyrethrins against whiteflies (*Bemisia tabaci*) and aphids (*Myzus persicae*). Pest Manag Sci. 2014; 70: 682-688.

75. Maheswaran R, Ignacimuthu S. Effect of confertifolin from *Polygonum hydropiper* L. against dengue vector mosquitoes *Aedes aegypti* L. Environ Sci Poll Res. 2015; 22: 8280-8287.

76. Corrêa DS, Tempone AG, Reimão JQ, Taniwaki NN, Romoff P, Fávero OA, Sartorelli P, Mecchi MC, Lago J. Anti-leishmanial and anti-trypanosomal potential of polygodial isolated from stem barks of *Drimys brasiliensis* Miers (Winteraceae). Parasitol Res. 2011; 109: 231-236.

Testings of *Myrtus communis* leaf extracts on mammalian and bacteria cells

7

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ABSTRACT

The aim of this work was the search of molecules biologically active from *Myrtus communis* leaves. Powders of dried leaves were extracted with different solvents (ethanol, ethanol/chloroform and chloroform) and after their evaporation the obtained powders were solubilized with a solution of 10% DMSO in water. After sterilization by filtering, we treated murine myeloma cells (P3X cell line) and bacteria (*Bacillus cereus*) for 24 hours with the obtained extracts. Subsequently, the ethanol/chloroform extract was processed by Thin Layer Chromatography (TLC). At the end of the run the silical-sheet was divided in three fractions (F1, F2, F3), each fraction tested on mammalian and bacterial cells and the F3 fraction resulted positive. Subsequently, by another TLC run the spots contained in F3 were separated and then tested on mammalian and bacterial cells. The spots (S3, S4, S5 and S6) contained in F3 fraction were assayed on P3X cells and *B. cereus*. After 24 hours of treatment, S3 did not show cytotoxic effect on P3X, S4 a weak activity whereas S5 and S6 showed cytotoxic effects, both with 50% of living cells; S3 and S4 showed antibacterial activity to *B. cereus* with ZOI of 11.2 mm and 12.8 mm respectively whereas no antibacterial activity was revealed in spot S5 and S6.The obtained results allowed to suppose that activities against P3X

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cells and *B. cereus* could be related to different molecules or molecule groups consisting the F3 fraction.

Keywords: *Myrtus communis*; *Bacillus cereus*; Thin Layer Chromatography (TLC).

1. INTRODUCTION

Plants contain many molecule families such as terpenes, terpenoids, phenols, flavonoids, anthraquinones and alkaloids which often show biological activities [1]. In past time the searching of biologically active plant compounds provided interesting results in the biomedical field and some useful molecules were identified and applied as active principles for pharmaceuticals [2-4].

Plants are also a source of antibiotics to human and animal pathogens [5] and such compounds could result useful for the increasing variety of new strains originated with the selection processes caused by the uncontrolled application of antimicrobial agents worldwide [6].

Myrtus communis L. (Fig. 1) is a plant widely used for food and beverage preparations and is well known in traditional medicine for its anti-inflammatory, antimicrobic and antioxidant properties [7]. It has been also shown that its essential oil showed antituberculotic effect to *Mycobacterium tuberculosis* [8] and acylphloroglucinol, a compound from *Myrtus* leaves, induces apoptosis in human fibroblasts [9].

Figure 1. *Myrtus communis* L.

In our continuous searching of new plant molecules having biological properties, here we report the results of our investigations carried out testing *Myrtus* leaf extracts on P3X murine myeloma cells, a mammalian cell line that we currently use as a model for evaluations of antiproliferative activity [10-12]. *Myrtus* leaf extracts were also tested on *Bacillus cereus*, an environmental bacterium responsible of gastroenteric infections [13].

2. MATERIALS AND METHODS

2.1. Plant materials and extraction procedure

Leaves were collected from *Myrtus communis* (1753) plants grown at the Botanical Garden of Tuscia University in Viterbo, Italy, and air dried for 90 days in the dark, until constant weight. Dried leaves were powdered using a mortar and the powder was stored at 4°C until use.

Three aliquots of 1 g of powder were used respectively for extraction with ethanol (E), with a 1:1 mixture of ethanol/chloroform (E/C) and with chloroform (C); solvents were added to the dried powder using a 1:4 (*w*/*v*) ratio. After incubating for 50 minutes in the dark at room temperature, samples were centrifuged at 14000 rpm (MIKRO 120,96 Hettich®,Tuttlingen, Germany) for 3 minutes and the pellet discarded; supernatants were then evaporated by a nitrogen flow and the residuals, after weighting, resuspended in 10% dimethyl sulfoxide (DMSO) in water to a concentration of 20 mg/ml; the obtained solutions were filtered by using 0.22 μm sterile filters. A negative control consisting of a 10% DMSO (cDMSO) solution in water was prepared in order to evaluate its possible toxic effects.

2.2. Cell cultures and cytotoxic activity

Cell line P3X63-Ag8.653 (P3X) was used as the model to test cytotoxic effects of *Myrtus communis* (MC) extracts. P3X cells, a non-secretory myeloma with a lymphoblastoid morphology, were cultured in Dulbecco's Modified Eagle's Medium (DMEM) complemented with fetal calf serum (FCS) to 10% and 2 mM glutamine at 37±1°C.

Possible cytotoxic activity of the extracts was tested by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays [14] on 96 well cell plates; 2×10^4 murine myeloma cells were plated in each well with 200 µl of culture medium containing the extract

to a final concentration of 0.1 mg/ml or containing fractions and TLC spots (see below) to a final concentration of 0.02 mg/ml. Some wells were added with medium containing cDMSO as negative control. After 24 hours of incubation, plates were centrifuged at 1300 rpm for 5 minutes, the exhaust medium was discarded and replaced with 100 µl of fresh medium supplemented with 0.5 mg/ml of MTT. After 3 hours and 30 minutes incubation in the dark, 100 µl of DMSO were added to each well and after 45 minutes, absorbance values were measured at 595 nm by a plate reader (Tecan Sunrise™).

2.3. Bacterial cultures and antibacterial activity

In the present investigation we used *Bacillus cereus* as the cellular model for testing possible antibacterial activity of *M. communis* leaf extracts. This strain is a member of the genus *Bacillus*, motile, sporogenous, aerobic/facultative anaerobic and Gram-positive. In our experiments, it was cultured on Plate Count Agar (PCA) at 30±1°C.

B. cereus was diluted to 0.15 OD, further diluted 1:100 and 100 µl of the solution containing bacteria plated on Petri dishes containing PCA. Antibacterial activities were tested using the standard agar disc diffusion method [15]. Five cotton disks (Blank Antimicrobial Susceptibility Disks, Oxoid™), four soaked with 15 µl of extract (20 mg/ml) or with isolated extract fractions (2 mg/ml) or with isolated extract spots (2 mg/ml), and in the center of plate one disk soaked with 15 µl of cDMSO (negative control) were placed inside each plate. After 24 hours of incubation the zone of inhibitions (ZOI) around the disks were measured in millimeters.

2.4. Thin Layer Chromatography

2.4.1. Identification of active fractions

Thin Layer Chromatography (TLC) analyses were performed to fractionate the MC ethanol/chloroform extract. To 1,5 cm from the bottom border of a silica plate (20 cm x 20 cm, Adamant UV_{254}, Macherey-Nagel GmbH & Co KG, Germany), 19 spots consisting of 5 µl of E/C (10 mg/ml) each one were loaded *via* Microliter 700 Series Syringes Hamilton (80500, Supelco, USA) at a distance of 1,5 cm between them. A 1:1 ethanol:chloroform solution (100 ml) was used as the mobile phase, chromatographic separation was stopped when the liquid front was 4 cm to the sheet top (approx. 2,5 ours). The sheet was divided into

3 fractions, denominated from the bottom F1, F2, F3. F1 was the most polar fraction and F3 was the less polar (Fig. 2). Silica fractions were scraped from the sheet and for desorption 5 ml of DMSO were added to each gram of silica powder; after 120 minutes of shaking, fractions were centrifuged at 14000 rpm for 5 minutes, silica powders were discarded and liquid phases completely dried under nitrogen stream.

Obtained powders were resuspended in 10% aqueous DMSO to a final concentration of 2 mg/ml. The samples were filtered using 0.22 μm sterile filters before assaying for biological activity. Solutions were then stored at 4°C.

2.4.2. Investigation of F3 fraction content

The chemical components of the F3 Fraction were further separated by preparative TLC techniques using the same conditions previously reported. At the end of the chromatographic separation, silica plates were viewed under UV lamps 254/368 (Ultraviolet Product Inc., USA) and some spots identified (Fig. 2). Selected spots were then processed as previously described.

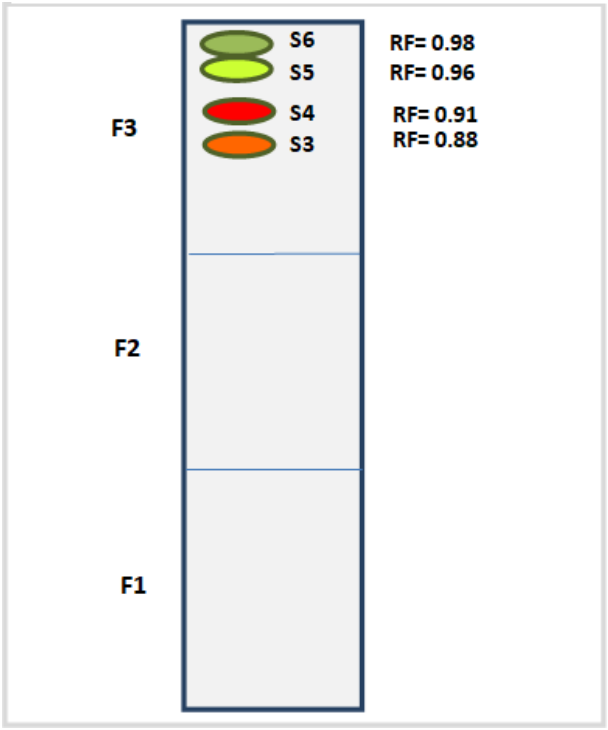

Figure 2. TLC pattern

2.5. Statistical analysis

All data were analyzed by one way ANOVA (STATISTICA 10, StatSoft) and significant differences between means at the 5% level were evaluated using the LSD Fisher test.

3. RESULTS AND DISCUSSION

During time *M. communis* plant was widely used in traditional medicine [16] and the aim of the present work is to contribute to enlarge the knowledge on compounds from the leaves of such plant which have biological activity. In our experimental work we carried out extraction processes using three different solvents in order to select compounds of different polarity.

The MTT assays carried out after treatment with the extracts (0.1 mg/ml) obtained by using different solvents, showed that cellular viabilities were around 50% and significantly different to control and cDMSO. E, E/C and C extracts showed similar results (Fig. 3).

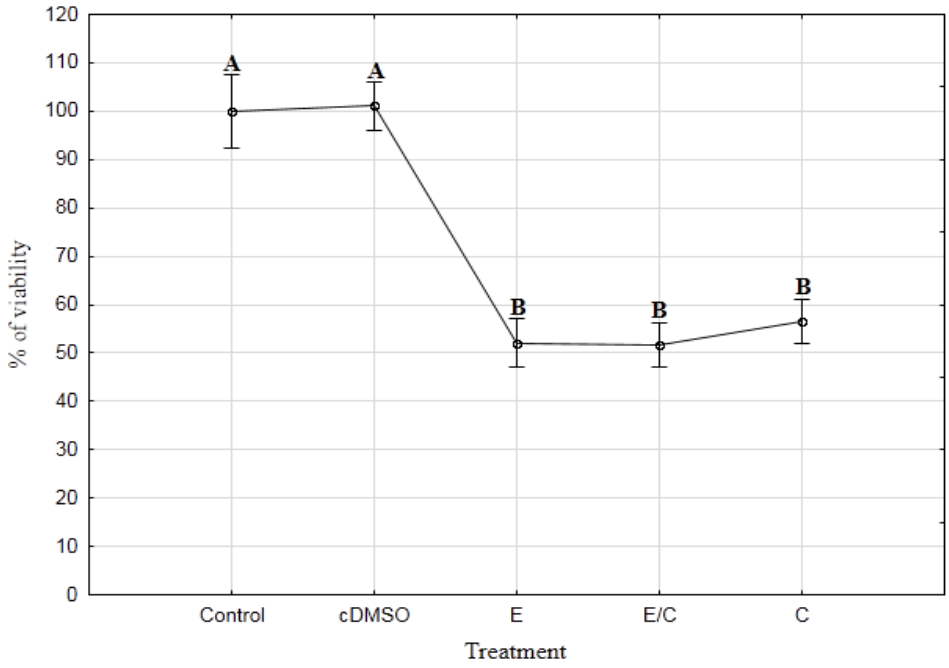

Figure 3. Percentage of P3X viability after 24 hours of treatment with the different extracts. Data are reported as means ± SEM (n = 6). Significant differences are represented by different letters (A-B= P<0.001)

Table 1. Summary of ZOI. Data were represented as mean of ZOI ± SEM

Treatment	ZOI (mm) ± SEM
cDMSO	0
E	16.6 ± 0.18
E/C	17.5 ± 0.19
C	17.5 ± 0.29
F1	0
F2	0
F3	6.1 ± 0.35
S3	11.2 ± 0.25
S4	12.8 ± 0.31
S5	0
S6	0

Antibacterial effects of *Myrtus* leaf extracts on *B. cereus* and other bacterial strains were already evaluated by Dulger and Gonuz [17]. They obtained the extract (200 mg/ml) through Soxhlet apparatus and soaked each disk with 50 µl of extract, obtaining 20 mm of ZOI.

In our work, we soaked disk with only 15 µl of each extract to 20 mg/ml of concentration and all the extracts showed antibacterial effects on *B. cereus* almost comparable with Dulger and Gonuz ZOI. E extract showed ZOI of 16.6 mm and both the others, E/C and C, showed a halo of 17.5 mm (Tab. 1, Fig. 6). Our results were in contrast with Irshad et al. [18] since they asserted that generally an ethanol extract has higher antibacterial properties than a chloroform extract; however, it should be noted that they assayed E and C extracts on bacterial strains such as *Lactobacillus subtilis*, *Bacillus thuringiensis*, *Corynebacterium*, *Escherichia coli* and not on *B. cereus*.

Considering that E/C and C extracts against *B. cereus*, showed the same biological activities but higher than E, E/C extract was investigated by TLC procedures. The activity of each fraction was assayed on P3X cells at the concentrations of 0.02 mg/ml and on bacterial cells of 2 mg/ml, respectively.

MTT assays carried out on P3X cells using F1, F2 and F3 fractions, showed that cytotoxic activity was present in F3 with a percentage of living cells of about 60% (Fig. 4); for bacterial testing, 15 µl of each fraction were assayed and a ZOI of 6.1 mm was detected in F3 fraction (Fig. 7).

Once F3 was identified as the biologically active fraction against our cell models, another TLC was run in order to identify the spots consisting the positive fraction and after observing the sheet through UV light, four spots were identified (Fig. 2).

The identified single spots (S3, S4, S5 and S6) were assayed on P3X and bacterial cells at the concentration of 0.02 mg/ml and 2 mg/ml respectively. After 24 hours of treatment, different levels of damage were showed by the identified single spots. On P3X cells, S3 did not show any cytotoxic effect while S4 only a weak activity (84% of living cells); S5 and S6 showed more prominent cytotoxic effects, both with 50% of living cells (Fig. 5); on the other hand, S3 and S4 showed ZOI of 11.2 mm and 12.8 mm respectively on *B. cereus* (Tab. 1, Fig. 8) whereas no antibacterial activity was revealed in spot S5 and S6. The negative control cDMSO did not show any biological effect on both P3X cells and bacterial strain *B. cereus*.

The obtained results allowed to suppose that activities against P3X and *B. cereus* could be related to different molecules or molecule groups consisting the F3 fraction.

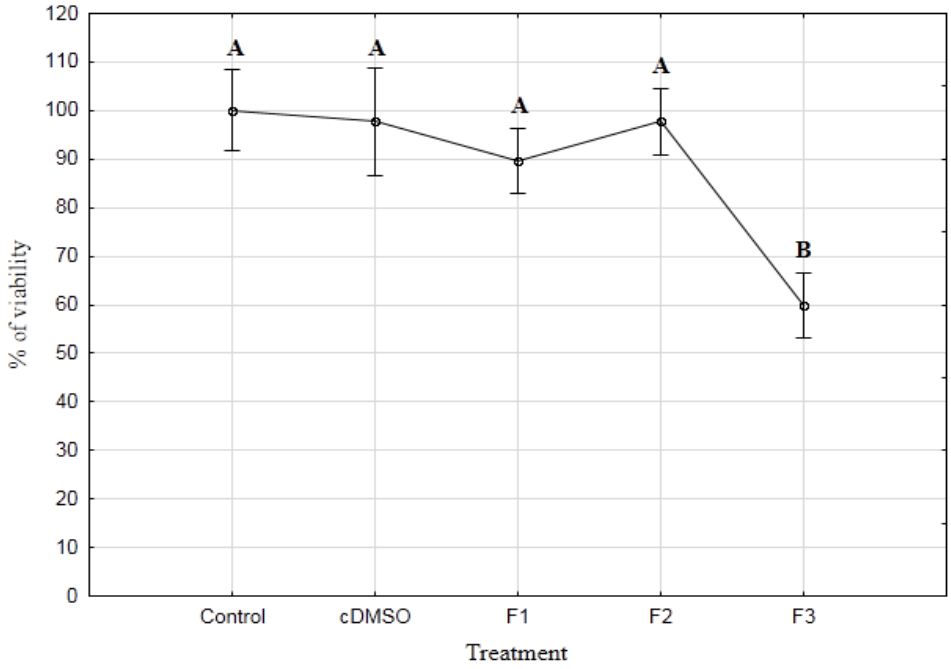

Figure 4. Percentage of P3X viability after 24 hours of treatment with the different fractions. Data are reported as means ± SEM (n = 6). Significant differences are represented by different letters (A-B= P<0.001)

In terms of chemical composition, myrtucommulone, a nonprenylated acylphloroglucinol, is contained in *M. communis* leaves [19-21], and several compounds were identified as belonging to myrtucommulones [22].

Some biological activities against cancer cell lines, as inductor of apoptosis were associated to myrtucommulone [9] and, in particular, Izgi and coworkers [23] showed that myrtucommulone isomer A is responsible of apoptosis induction.

Alipour et al. [24] in its review attributed the strong antibacterial activities to monoterpenes such as α-pinene, limonene, eucalyptol, linalool and terpineol, present in high quantity in *M. communis* leaves. Appendino et al. [25] investigated antibacterial activity of a polar glycosidic fraction of *M. communis* leaves and attributed antibacterial action to gallo-myrtucommulone. It would be interesting to evaluate if the biological activities we reported here are related to myrtocommulone and in this view further investigations are running in our laboratory.

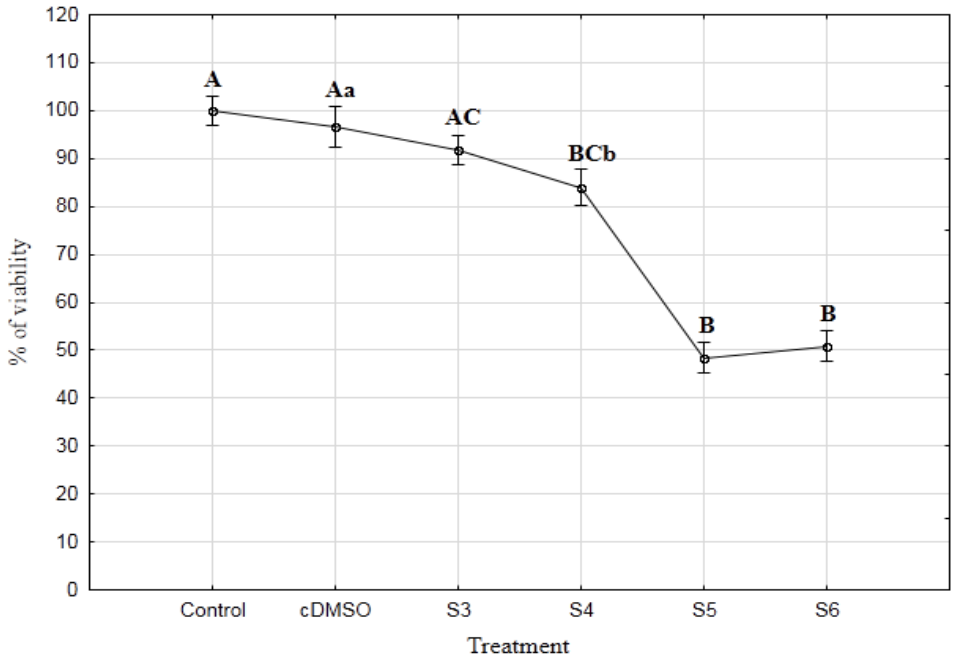

Figure 5. Percentage of P3X viability after 24 hours of treatment with the different spots. Data are reported as means ± SEM (n = 6). Significant differences are represented by different letters (a-b = $P < 0.05$; A-B= $P<0.001$)

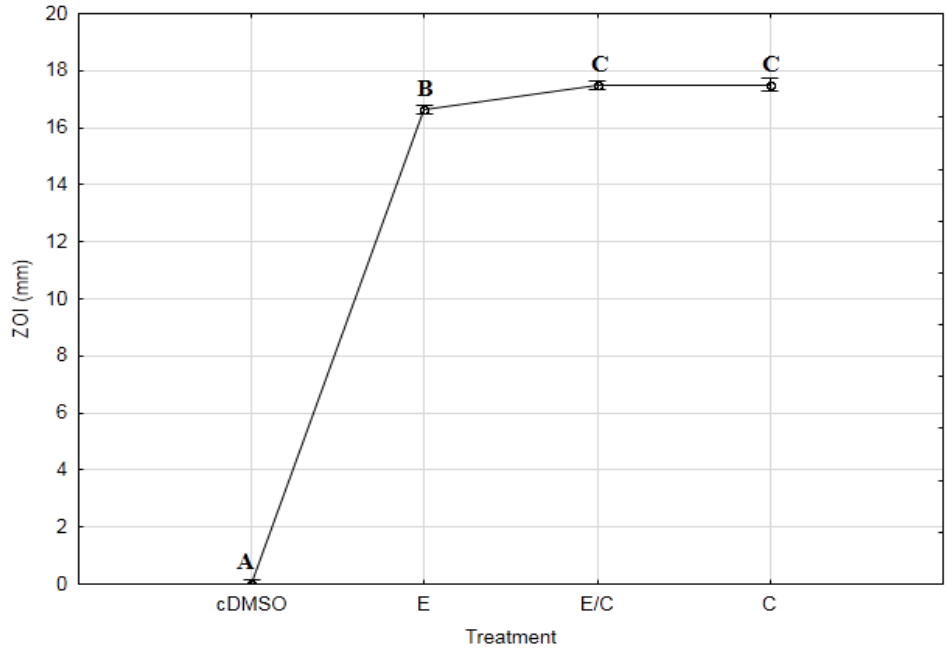

Figure 6. ZOI (mm) measured after 24 hours of treatment with the different extracts. Data are reported as means ± SEM (n = 6). Significant differences are represented by different letters (A-C = P < 0.001)

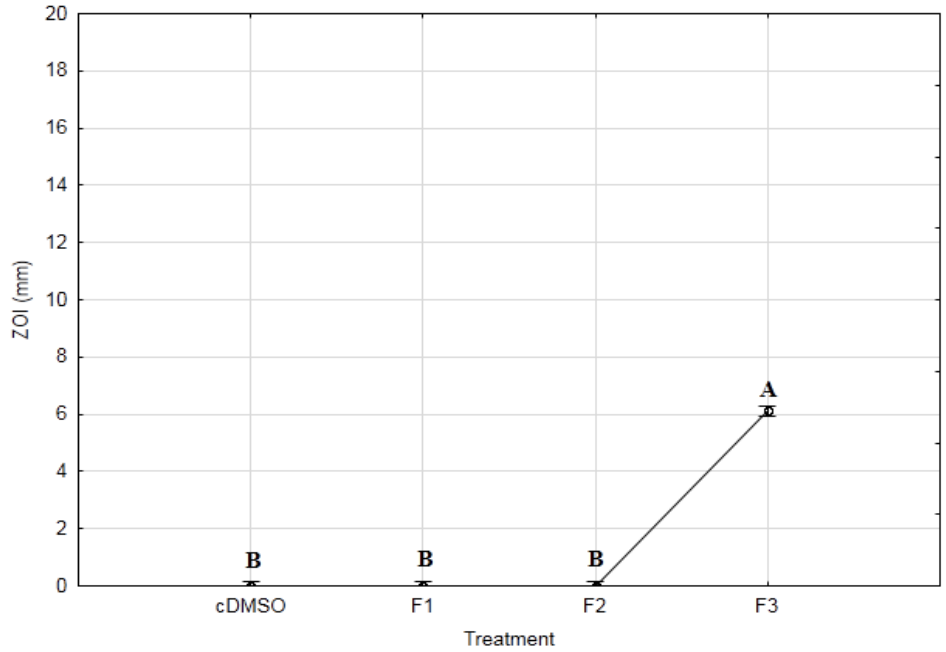

Figure 7. ZOI (mm) measured after 24 hours of treatment with the different fractions. Data are reported as means ± SEM (n = 6). Significant differences are represented by different letters (A – B = P < 0.001)

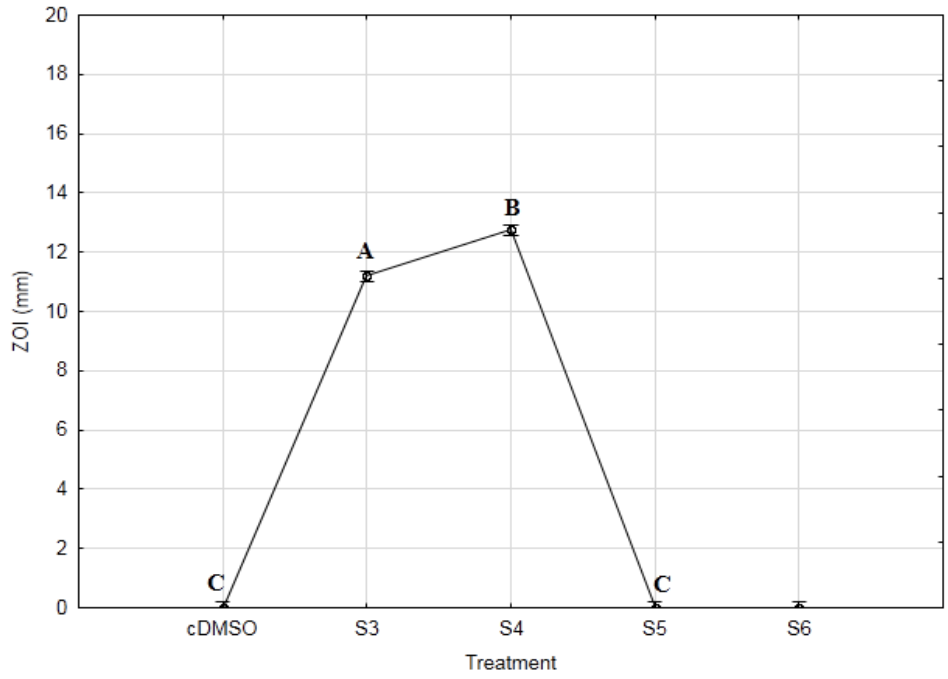

Figure 8. ZOI (mm) measured after 24 hours of treatment with the different spots. Data are reported as means ± SEM (n = 6). Significant differences are represented by different letters (A – C = P < 0.001)

4. CONCLUSIONS

All leaf extracts showed cytotoxic activity to P3X cells and showed also antibacterial activity against *B. cereus*. TLC F3 fraction contained 4 active spots: S5 and S6 resulted active against P3X murine myeloma cells and S3 and S4 on *Bacillus cereus* bacterial strain.

Further biological and chemical investigations are needed to identify and characterize the molecules responsible for both antiproliferative and antibacterial activities.

REFERENCES

1. Sasidharan S, Chen Y, Saravanan D, Sundram KM, Latha LY. Extraction, isolation and characterization of bioactive compounds from plants' extracts. Afr J Tradit Complem Altern Med. 2011; 8(1): 1-10.
2. Triggiani D, Ceccarelli D, Tiezzi A. From plants to pharmaceuticals. In: Plant molecules, basic and applied research. Tiezzi A, Santucci A, eds. Casa Editrice Gangemi, Roma, 2006, pp. 163-176.

3. Mishra BB, Tiwari VK. Natural products: an evolving role in future drug discovery. Eur J Med Chem. 2011; 46: 4769-4807.
4. Newman DJ, Cragg GM. Natural products as sources of new drugs from 1981 to 2014. J Nat Prod. 2016; 79: 629-661.
5. Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev. 1999; 12(4): 564-582.
6. Rodríguez-Noriega E, León-Garnica G, Petersen-Morfín S, Pérez-Gómez HR, González-Díaz E, Morfín-Otero R. Evolution of bacterial resistance to antibiotics in México, 1973-2013. Biomedica. 2014; 34(Suppl 1): 181-9020.
7. Messaoud C, Laabidi A, Boussaid M. *Myrtus communis* L. infusions: the effect of infusion time on phytochemical composition, antioxidant, and antimicrobial activities. J Food Sci. 2012; 77(9): C941-947.
8. Zanetti S, Cannas S, Molicotti P, Bua A, Cubeddu M, Porcedda S, Marongiu B, Sechi LA. Evaluation of the antimicrobial properties of the essential oil of *Myrtus communis* L. against clinical strains of *Mycobacterium* spp. Interdisc Persp Inf Dis. 2010: 931530.
9. Tretiakova I, Blaesius D, Maxia L, Wesselborg S, Schulze-Osthoff K, Cinatl J, Michaelis M, Werz O. Myrtucommulone from *Myrtus communis* induces apoptosis in cancer cells via the mitochondrial pathway involving caspase-9. Apoptosis. 2008; 13(1): 119-131.
10. Mamadalieva NZ, Egamberdieva D, Zhanibekov AA, Triggiani D, Tiezzi A. Chemical components of *Silene viridiflora* and their biological properties. Chem Nat Comp. 2009; 45(4): 589-591.
11. Ceccarelli D, Ovidi E, Triggiani D, Morelli C, Speranza G, Taddei A, Tiezzi A. Antiproliferative activity of *Aloe arborescens* leaf skin extracts tested on murine myeloma cells: Cytological studies and chemical investigations. Med Arom Plant Sci Biotechnol. 2012; 6: 31-36.
12. Mamadalieva NZ, Mamedov NA, Craker LE, Tiezzi A. Ethnobotanical uses and cytotoxic activities of native plants from the Lamiaceae family in Uzbekistan. Acta Horticult. 2012; 1030: 61-70.
13. Kotiranta A, Lounatmaa K, Haapasalo M. Epidemiology and pathogenesis of *Bacillus cereus* infections. Microbes Infect. 2000; 2(2): 189-198.
14. Van Meerloo J, Kaspers GJ, Cloos J. Cell sensitivity assays: the MTT assay. Cancer cell culture: methods and protocols. Methods Mol Biol. 2011; 731: 237-245.
15. Das K, Tiwari RKS, Shrivastava DK. Techniques for evaluation of medicinal plant products as antimicrobial agents: current methods and future trends. J Med Plants Res. 2010; 4(2): 104-111.

16. Sumbul S, Ahmad MA, Asif M, Akhtar M. *Myrtus communis* Linn. - A review. Indian J Nat Prod Resour. 2011; 2(4): 395-402.
17. Dulger B, Gonuz A. Antimicrobial activity of certain plants used in Turkish traditional medicine. Asian J Plant Sci. 2004; 3(1): 104-107.
18. Irshad S, Mahmood M, Perveen F. In vitro antibacterial activities of three medicinal plants using agar well diffusion method. Res J Biol. 2012; 2(1): 1-8.
19. Kashman Y, Rotstein A, Lifshitz A. The structure determination of two new acylphloroglucinols from *Myrtus communis* L. Tetrahedron. 1974; 30: 991-997.
20. Appendino G, Bianchi F, Minassi A, Sterner O, Ballero M, Gibbons S. Oligomeric acylphloroglucinols from myrtle (*Myrtus communis*). J Nat Prod. 2002; 65(3): 334-338.
21. Koeberle A, Pollastro F, Northoff H, Werz O. Myrtucommulone, a natural acylphloroglucinol, inhibits microsomal prostaglandin E2 synthase-1. Brit J Pharmacol. 2009; 156(6): 952-961.
22. Müller H, Paul M, Hartmann D, Huch V, Blaesius D, Koeberle A, Werz O, Jauch J. Total synthesis of myrtucommulone A. Angewandte Chemie International Edition, 2010; 49(11), pp. 2045-2049.
23. Izgi K, Iskender B, Jauch J, Sezen S, Cakir M, Charpentier M, Canatan H, Sakalar C. Myrtucommulone-A induces both extrinsic and intrinsic apoptotic pathways in cancer cells. J Biochem Mol Toxicol. 2015; 29(9): 432-439.
24. Alipour G, Dashti S, Hosseinzadeh H. Review of pharmacological effects of *Myrtus communis* L. and its active constituents. Phytother Res. 2014; 28(8): 1125-1136.
25. Appendino G, Maxia L, Bettoni P, Locatelli M, Valdivia C, Ballero M, Stavri M, Gibbons S, Sterner O. Antibacterial galloylated alkylphloroglucinol glucosides from Myrtle (*Myrtus communis*). J Nat Prod. 2006; 69(2): 251-254.

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