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Article

Phytochemical, Technological and Pharmacological Study on the Galenic Dry Extracts Prepared from German chamomile (*Matricaria chamomilla* L.) Flowers

Janne Sepp [1], Oleh Koshovyi [1,2], Valdas Jakstas [3], Vaidotas Žvikas [3], Iryna Botsula [2], Igor Kireyev [2], Karina Tsemenko [2], Oleksandr Kukhtenko [2], Karin Kogermann [1], Jyrki Heinämäki [1] and Ain Raal [1,*]

[1] Institute of Pharmacy, Faculty of Medicine, University of Tartu, Nooruse 1, 50411 Tartu, Estonia; e-mail: oleh.koshovyi@ut.ee (O.K.); jyrki.heinamaki@ut.ee (J.H.); ain.raal@ut.ee (A.R.)

[2] National University of Pharmacy; 53 Pushkinska str., Kharkiv, 61002, Ukraine; e-mail: oleh.koshovyi@gmail.com (O.K.); ivkireev@ukr.net (I.K.); botsula.iv@gmail.com (I.B.); k-cemenko@ukr.net (K.T.); kukhtenk@gmail.com (O.Ku.)

[3] Institute of Pharmaceutical Technologies, Lithuanian University of Health Sciences, 44307 Kaunas, Lithuania; e-mail: Valdas.Jakstas@lsmu.lt (V.J.); vaidotas.zvikas@lsmu.lt (V.Z.)

* Correspondence: Ain Raal, e-mail: ain.raal@ut.ee; Tel.: +372 5027574, Nooruse 1, 50411 Tartu, Estonia

Abstract: German chamomile galenic preparations are used to treat mild skin diseases, inflammation and spasms, and they have also reported to have anxiolytic and sedative effects. The medicinal use of chamomile is well-known in ethnomedicine. Since chamomile has shown to have some adverse effects, there is a need to develop new formulations, such as dry extracts, for the present herb. The aims of this study were (1) to develop a novel method for preparing essential oils and dry extracts from German chamomile flowers, (2) to reveal the phytochemical composition of such extracts, and (3) to verify the analgesic and soporific activity of extracts *in vivo* with a rodent animal model. Furthermore, our study aims to gain knowledge of the ethnomedical status of chamomile in the past and presence. Essential oils and dry extracts were successfully prepared from the tincture and aqueous extracts obtained after hydrodistillation and from a tincture powder. Total 22 phenolic compounds (7 hydroxycinnamic acids, 13 flavonoids and 2 phenolic acids), were found in the dry extracts by using UPLC-MS/MS. Total 9 main terpenoids were identified in the chamomile oil, and these terpenoids represent the chemotype rich in bisabolol oxides A and B. In the highest yield, the ratio of phenolic compounds and extract was in the range of 1:14-1:16 and triple extraction was performed. In *in-vivo* studies with mice and rats, the extracts showed analgesic activity and improvements in sleep. The highest sedative effect in rodents was found with the extract prepared by using a 70% aqueous ethanol solution for extraction. The administration of such extract at the dose of 50 mg/kg significantly prolonged the sleeping time in rats.

Keywords: German chamomile; flower; galenic extract; extraction; analgesic activity; soporific effect

1. Introduction

Since ancient times German chamomile has been used in both folk and official medicine. Chamomile (*Matricaria chamomilla* L.) belongs to the family *Asteraceae* and it is an essential oil-content medicinal herb widely known and used in Europe, Asia and America [1–3]. Chamomile is usually consumed as tea or tincture. Essential oil and tincture are the components of several traditional and homeopathic medicinal drugs [1,4]. To date, the galenic preparations of German chamomile have been used to treat mild skin diseases, inflammation, and spasms, and such preparations have also been reported to have anxiolytic and sedative effects [1]. They are also useful in treating many other diseases and disorders, such as flatulence, colic, ulcers, wound healing, hysteria, depression, etc. [1].

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In 2000, chamomile was assigned as an over-the-counter (OTC) dietary supplement by the US FDA. In addition, German chamomile and its essential oil, extracts, and distillates are stated as generally regarded as safe for use in food products [1]. The national pharmacopoeia of total 26 countries recognizes chamomile as a drug [1,4]. Since chamomile is widely used for the treatment of various diseases, its pharmaceutical and medicinal value can not be ignored. Therefore, it is worth developing novel pharmaceutical formulations (galenic preparations) for chamomile to further improve the medicinal use and efficacy of the present medicinal herb.

Galenic preparations and dry raw materials of German chamomile are widely applied as medicines. One of the most common medicinal preparation of the present herb is chamomile tincture. The method for the preparation of chamomile tincture is well known [5]. However, the major limitation of such tincture is poor chemical stability and, consequently, the changes in the pharmacodynamics of actives in medicinal uses. In addition, ethanol is used as an extracting solvent in preparing chamomile tinctures, thus excluding many patient groups who can not use this medicinal product (i.e., children, pregnant women, nursing mothers, and persons whose activities require increased attention, etc.). Ethanol can also directly affect the central nervous system (CNS) and modulate the effects of the active ingredients of the present medicinal herb. Therefore, chamomile decoction and tea are recommended for the abovementioned patients. Decoction and tea, however, also have limitations in medicinal uses since they are non-standardized preparations. Furthermore, the preparation of both decoction and tea takes a long time, and the chemical composition of such liquid preparations can change during storage (a limited storage time). Therefore, there is a true demand for new galenic preparations (dry extracts) to advance the medicinal use chamomile herb.

In our days, the global natural resources are becoming limited. Therefore, the interest in the development and preparation of new plant-origin materials and products for medicinal uses is steadily growing. This approach makes it possible to extend the selection of medicines, to use natural resources rationally, to increase the profitability of pharmaceutical companies, and to reduce the negative impact of pharmaceutical production on the environment [6–8]. For preparing a chamomile tincture, one-time (single) extraction is used [5], and there is still a significant amount of biological active substances (BAS) in the wastes. Therefore, it is important to find out and use the optimal process parameters (such as an extraction frequency) in the extraction of BAS from a chamomile herb. This is also valid for the isolation and preparation of chamomile essential oil by means of hydro-distillation. The waste of distillation extract and chamomile flower powder contains a significant amount of BAS. Thus, the development of standardized dry extracts of German chamomile flowers is still one of the key topic areas in the pharmaceutical formulation research of the present medicinal herb.

The aim of the present study was three-fold: (1) to develop novel methods for preparing essential oils and dry extracts from German chamomile flowers, (2) to disclose the phytochemical composition of such dry extracts, and (3) to verify the analgesic and soporific activity of extracts *in vivo* with mice and rats. Moreover, the study gains knowledge of the ethnomedical status of chamomile in the past and present.

2. Results

2.1. Ethnomedical study

In the data of the Estonian Literary Museum folklore archive, there were the total of 150 index cards in the catalogue of chamomiles. Unfortunately, it was not possible to distinguish the two species (German chamomile and pineapple weed) of the genus *Matricaria* (chamomile), which have been and still are common plants in Estonia. Therefore, both species of the genus are discussed here together. The materials analyzed date back to 1891, thus covering almost a period of 100 years (the latest entry is from 1989). All Estonian counties were represented (n=15) in the study according to current administrative division. Almost 1/3 of the records (index cards) did not specify the medical use of chamomiles and the same proportion referred to the treatment of respiratory tract diseases.

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According to the index cards, chamomiles were used for relieving ocular diseases (15.3%), inflammation (6.0%) and trauma (5.3%) (Table 1). Chamomiles were also used for treating pain, infectious diseases and sedative effects, and for relieving spasms. There were single references about the other diseases, which were listed under the category "Other". It is worth to mention that two out of three descriptions of the use of chamomiles as sedative purposes, were related to the treatment of children:

"Chamomile tea was given to children against crying." (ERA II 201, 105 (67), written down by Lepp, K., 1938, Saare county, Karja, Leisi municipality)

"If the children scream a lot, chamomile tea is given to the children to drink." (E57311(29), written down by Eisen, M. J., year unknown, Rapla county, Vigala)

"Chamomile was collected for tea. A sick person was given chamomile tea for sedation." (KKI, KS, Jõulmaa, H., 1977, East-Viru county, Iisaku, Uhe)

The most common route of administration of chamomiles was an external (40.7%) route (an internal route 36.0%). The route of administration was not specified in 12.6% of the references, or alternatively both routes of administration were mentioned (10.7%).

Table 1. The use of genus *Matricaria* plants in folk medicine in Estonia - indications.

Category	Number of records	Percentage of total, %
Unspecified	45	30.0
Respiratory tract diseases	45	30.0
Ocular diseases	23	15.3
Inflammation	9	6.0
Trauma	8	5.3
Pain	5	3.3
Infection diseases	5	3.3
Other	5	3.3
Sedative	3	2.0
Spasms	2	1.3

2.2. Phytochemical composition of dry extracts and essential oil

The dry extracts of German chamomile were light powders with a light to dark brown color and a specific smell. The loss on drying (LOD) values for the extracts ranged from 4.3% to 5.0%.

The main phenolic compounds of the dry extracts were identified and quantified by UPLC-MS/MS (Table 2). For the assay of phenolic compounds, hydrocinnamic acids and flavonoids, the established pharmacopeia spectrophotometric methods were used. Total 22 phenolic compounds (7 hydroxycinnamic acids, 13 flavonoids and 2 phenolic acids) were found in the dry extracts of German chamomile (Table 2).

Table 2. Content of phenolic compounds in the German chamomile extracts.

Substance	Content in the extract		
	G1	G2	G3
UPLC-MS/MS, µg/g of dry extract			
Neochlorogenic acid	1672.30 ± 85.39	444.94 ± 20.16	441.14 ± 13.32
Luteolin	83.99 ± 12.65	310.93 ± 22.73	74.87 ± 3.871
Isoquercitrin	477.46 ± 68.82	921.16 ± 85.20	42.15 ± 14.12
Cryptochlorogenic acid	16.51 ± 2.42	80.74 ± 13.48	0
Luteolin-4-O-glucoside	16.98 ± 2.86	45.11 ± 3.67	0
Chlorogenic acid	3930.89 ± 224.37	11742.31 ± 376.34	1280.86 ± 98.96
Quercetin	18.87 ± 1.20	172.15 ± 12.01	9.37 ±1.03
Isorhamnetin-3-O-rutinoside	9.34 ± 0.56	15.40 ± 1.60	0
Isorhamnetin-3-glucoside	257.7 ± 27.04	410.75 ± 52.07	46.43 ± 4.38

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Luteolin-3,7-diglucoside	18.11 ± 4.59	20.72 ± 1.88	0
Vanilic acid	175.96 ± 13.28	86.58 ± 5.54	71.59 ± 7.22
Caffeic acid	42.98 ± 3.82	43.04 ± 3.22	33.46 ± 2.95
3,4-Dihydroxyphenylacetic acid	376.15 ± 27.,47	184.05 ± 13.38	159.71 ± 12.16
Isorhamnetin	17.99 ± 1.89	125,32 ± 12.71	15.51 ± 2.27
Apigenin	84.93 ± 4.88	578.65 ± 63.91	12.98 ± 2
Kaempherol-3-O-glucoside	34.79 ± 1.46	50.76 ± 2.10	0
Rutin	45.34 ± 5.55	126.49 ± 5.73	0
Hyperoside	224.42 ± 21.56	366.82 ± 21.21	65.15 ± 2.36
Luteolin-7-O-glucoside	616.65 ± 63.46	1061.82 ± 83.68	123.64 ± 31.82
4.5-Dicaffeoylquinic acid	3565.27 ± 266.90	4912.17 ± 416.85	541.70 ± 26.44
3.5-Dicaffeoylquinic acid	1823.72 ± 136.53	2512.69 ± 213.23	277.09 ± 13.52
3.4-Dicaffeoylquinic acid	3739.46 ± 279.94	5152.17 ± 437.22	568.17 ± 27.73
Spectrophotometry, %			
Phenolic compounds	6.19 ± 0.29	9.70 ± 0.52	2.27 ± 0.11
Hydrocinnamic acids	1.57 ± 0.09	3.47 ± 0.15	0.21 ± 0.01
Flavonoids	3.63 ± 0.11	9.92 ± 0.32	0.45± 0.01

Notes: G1 – the dry water extract after distillation of essential oil; G2 – the dry extract obtained with 70% of ethanol solution; G3 – the dry water extract after obtaining tincture.

Table 3 lists the main terpenoids and their content in the German chamomile essential oil obtained by a hydro-distillation method. Total nine (9) terpenoids were identified and quantified representing 97.3% of the composition of essential oil.

Table 3. The content of principal terpenoids in the German chamomile essential oil.

RI (DB-5)	Compound	Content in the oil, %	References [9–11]
1455	(E)-ß-Farnesene	4.30	1450-1456 [9–11]
1570	Spathulenol	2.24	1568-1570 [9–11]
1649	α-Bisabolol oxide B	22.00	1648-1649 [9–11]
1656	β-Bisabolol	1.21	1640-1656 [9–11]
1673	α-Bisabolone oxide A	15.92	1670-1674 [9–11]
1740	α-Bisabolol oxide A	44.01	1734-1741 [9–11]
1876	cis-Enyne-bicycloether	4.13	1867-1876 [9–11]
1978	Hexadecanoic acid	1.70	1977 [11]
2143	cis-Linoleic acid	1.79	2143 [11]
	In total	97.30	

2.3. Optimization of a dry extract G2 preparation

For optimizing an extraction process, the identification and setting of the levels of critical material and process parameters are of primary importance. Such parameters could be, e.g., the nature and concentration of an extractant, extraction conditions (temperature, speed and time, and kind of extraction), the ratio of an extractant to raw material, etc. These parameters affect not only the pharmaceutical quality of the product but also the manufacturing costs and, subsequently, the cost of a final medicinal product. For example, the ineffective use of extractants can significantly increase the formulation-related challenges in pharmaceutical development and reduce the therapeutic effect of the medicinal product.

To determine an effective ratio of extractant to German chamomile flowers in preparing a chamomile tincture, the yield of extractive substances and BASs (phenolic compounds, hydroxycinnamic acids and flavonoids) was studied based on DIR (the ratio of extractant to raw materials) and the multiplicity of extraction. For this study, the maceration was performed by using 70% aqueous ethanol solution and conducting total six (6) sequential stages of extraction. The volume of ethanol used in the extraction was varied to optimize the preparation of a chamomile tincture. The

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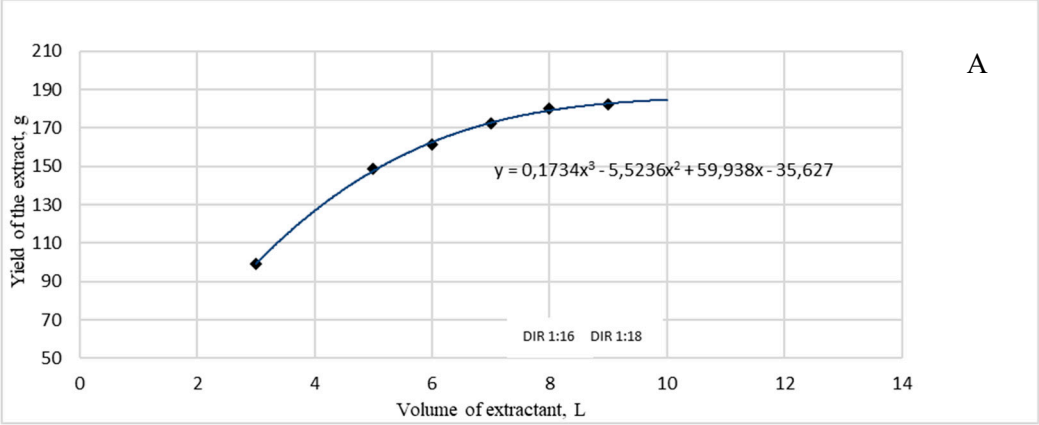
study was conducted at an ambient room temperature under normal pressure conditions using a laboratory percolator. In the liquid extracts, the content of extractive substances and BASs (phenolic compounds, hydroxycinnamic acids and flavonoids) was determined using pharmacopeia methods (Table 3). Total 500.0 g of German chamomile flowers was used as a plant material. The coefficient for the absorption of extractant was 2.04 (chamomile flowers in a 70% aqueous ethanol solution). The results on the extractive substance contents in a dry residue, are summarized in Table 4.

Table 4. Dynamics of phenolic compounds extraction with 70% aqueous ethanol solution from German chamomile flowers.

Extraction stage	Dry residue, %	Content (%) in the dry residue		
		Phenolic compounds	Hydrocinnamic acids	Flavonoids
1	5.00 ± 0.28	9.13 ± 0.34	2.46 ± 0.12	6.12 ± 0.19
2	2.57 ± 0.13	7.80 ± 0.10	3.24 ± 0.17	7.89 ± 0.04
3	1.4 ± 0.06	8.29 ± 0.27	3.79 ± 0.23	6.89 ± 0.26
4	1.1 ± 0.08	4.39 ± 0.24	1.91 ± 0.11	2.53 ± 0.04
5	0.8	2.85 ± 0.13	0.72 ± 0.04	0.58 ± 0.03
6	0.2	1.35± 0.05	0.99 ± 0.03	0.28 ± 0.01

Based on the phytochemical results (shown in Table 4), the yield of extractive substances and BASs (phenolic compounds, hydroxycinnamic acids and flavonoids) was used as a major criterion for optimizing a chamomile extraction process. For optimizing a BAS extraction rate, the mass yield coefficient of each stage ($m_{i\,BAS}/V_{i\,extractant}$) was calculated for each of the indicators with a "Statistika" software. The dependence of these factors on the extraction rate was derived to determine a rational extraction rate (Figure 1) [12–14].

The effects of DIR and the multiplicity of extraction on the content of extractive substances (yield) was determined. Figure 1 shows the effects of a DIR ratio on the yield of extractive substances and BASs (phenolic compounds, hydroxycinnamic acids and flavonoids) in the extraction of German chamomile flowers with a 70% aqueous ethanolic solution. Polynomial equations of the dependence between the yield of the BAS and the ratio of the extractant to raw material, were generated and used for the optimization.



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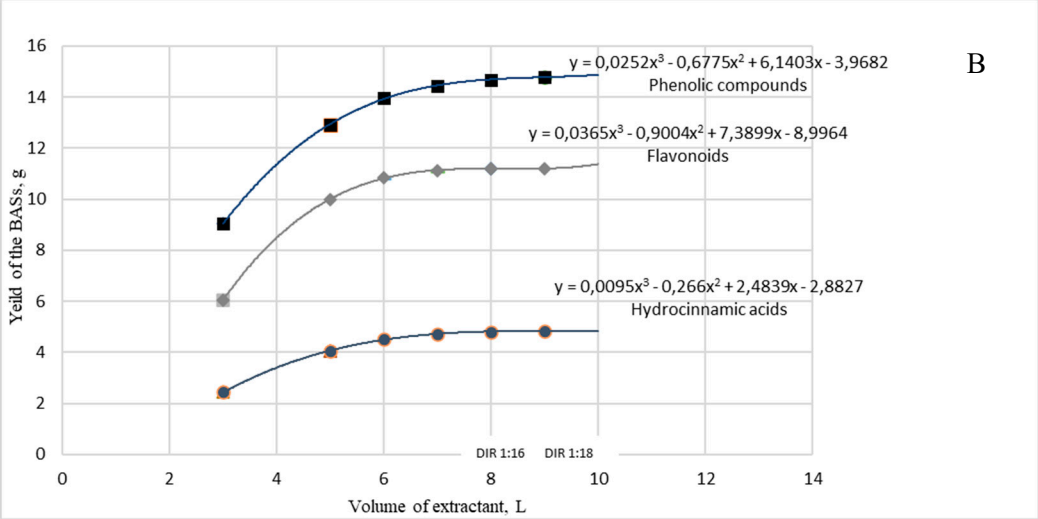

Figure 1. The effect of a DIR ratio on the yield of extractive substances (A) and BASs (B) in the extraction of German chamomile flowers.

2.4. Pharmacological study on analgesic and soporific activity

2.4.1. Analgesic activity

The galenic preparations of chamomile are widely used for the treatment of e.g., inflammatory skin diseases and dental disorders due to their anti-inflammatory and analgesic effects. In the present study, the analgesic activity of chamomile dry extracts was verified with a hot-plate test.

Table 5 shows the results of a hot-plate test with mice confirming the analgesic effect of the German chamomile extracts studied. The administration of dry extracts G1 and G3 slightly increased the reaction time of mice to the thermal stimulus. A higher analgesic effect was observed with the mice administered with a dry extract G2 (at all three doses studied) compared to a reference mice group, and the mice received acetaminophen.

Table 5. Analgesic activity of the German chamomile extracts in mice (n= 6).

Agent	Group	Dose, mg/kg	The time of discomfort occurrence (seconds) / Analgesic activity (%) in relation to [control] and (reference drug) after administration in				
			30 min	60 min	120 min	180 min	240 min
Intact animals	1		7.20±0.29	7.10±0.61	7.08±0.27	7.15±0.65	6.73±0.94
Extract G1	2	25	7.85±0.39 / [9%] (-25%) *	8.48±0.39 / [19%] (-18%) *	8.60±0.34 / [21%] # (-18%) *	7.97±0.21 / [11%] (-16%)	7.67±0.34 / [14%] (-8%)
	3	50	9.65±0.45 / [34] # (-8%)	9.83±0.53 / [38%] # (-5%)	9.63±0.50 / [36%] # (-9%)	8.77±0.27 / [23%] (-7%)	8.23±0.28 / [22%] (-1%)
	4	100	9.80±0.59 / [36%] # (-6%)	10.13±0.61/ [43%] # (-2%)	9.97±0.59 / [41%] # (-5%)	9.32±0.57 / [30%] # (-1%)	8.28±0.37 / [23%] (-1%)
Extract G2	5	25	9.63±0.54 / [34%] # (-8%)	9.97±0.60 / [40%] # (-4%)	8.65±0.48 / [22%] # (-18%)	7.98±0.12 / [12%] (-16%)	8.43±0.21 / [25%] (1%)

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	6	50	11.43±0.85 / [59%] # (9%)	11.83±0.77 / [67%] # (14%)	11.72±0.73 / [65%] # (11%)	11.13±0.73 / [56%] # (18%)	8.67±0.31 / [29%] (4%)
	7	100	12.50±0.36 / [74%] # (20%) *	12.52±0.31 / [76%] # (21%) *	12.47±0.30 / [76%] # (18%) *	9.63±0.50 / [35%] # (2%)	9.02±0.39 / [34%] (8%)
Extract G3	8	25	8.32±0.39 / [16%] # (-20%) *	8.88±0.31 / [25%] (-14%)	8.30±0.17 / [17%] # (-21%) *	8.13±0.30 / [14%] (-14%)	7.72±0.35 / [15%] (-8%)
	9	50	7.80±0.48 / [8%] (-25%) *	8.33±0.45 / [17%] (-20%)	8.33±0.37 / [18%] (-21%)	7.95±0.34 / [11%] (-16%)	7.70±0.24 / [14%] (-8%)
	10	100	8.80±0.64 / [22%] (-16%)	8.88±0.65 / [25%] (-14%)	8.67±0.55 / [22%] (-18%)	8.38±0.54 / [17%] (-11%)	8.28±0.51 / [23%] (-1%)
Acetaminophen	11	50	10.54±0.73	10.38±0.62	10.53±0.74	9.45±0.60	8.35±0.36

* Statistical significant ($p < 0.05$) in comparison to the group of acetaminophen 50 mg/kg (Student's test). # Statistical significant ($p < 0.05$) in comparison to the group of intact animals (Student's test).

2.4.2. Soporific activity

Chamomile tea has been used for centuries in sedative applications and for improving a sleep. In our study, the soporific activity of chamomile dry extracts was studied with rats by determining the duration of sleep (i.e., the time while the rats were in a lateral position) after the administration of the dry extracts and thiopental sodium. The results of soporific activity are summarized in Table 6. The administration of German chamomile extracts 20 minutes before a thiopental sodium intake induced a prolonged sleep effect inrats. With the animal group given the extract G2 (at the dose of 50 mg/kg) prior to the administration of thiopental sodium, the sleeping time was prolonged 117.3% compared the animal group given thiopental sodium (40 mg/kg) only. This suggests the sedative effect of the present dry extract of chamomile.

Table 6. Impact of the extracts G1, G2, and G3 on the duration of thiopental sleep, t±Δt (n = 6).

Agent	Group	Dose, mg/kg	Average duration of a sleep, min	Soporific effect, %
Control group	1	40	104.83±8.76	100%
Extract G1	2	25	140.33±6.52*#	133.9%
	3	50	201.83±4.69*	192.5%
	4	100	148.83±3.88*#	142.0%
Extract G2	5	25	186.33±6.12*	177.7%
	6	50	227.83±7.59*	217.3%
	7	100	190.00±6.97*	181.2%
Extract G3	8	25	177.83±4.00*#	169.6%
	9	50	136.83±4.74*#	130.5%
	10	100	166.33±9.93*#	158.7%
Valerian extract	11	2.15	204.17±8.39	194.8%

*Statistical significant ($p < 0.05$) in comparison to the group received sodium thiopental (Student's test). #Statistical significant ($p < 0.05$) in comparison to the group received "Valerian syrup AN NATUREL" (Student's test).

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3. Discussion

Our previous works have shown ethnomedicinal traditions as a valuable and inspired source of ideas for pharmaceutical studies [15–17]. Total three folkloristic descriptions have been found in the ethnomedicine database of Estonia on the use of chamomile tea as a sedative aid [18,19]. Interestingly, two of these descriptions are related to the children stating the following indications: "to children against crying" and "if the children scream a lot". The reason for crying and/or screaming may be a meteorism after feeding children. On the other hand, the Historical Estonian Folk Medicine Botanical Database ("Herba") [20] shows that the main sedative plants used in Estonia are chamomile, valerian, and lime flowers. The "Herba" database also suggests: "If there is a lack of sleep, drink chamomile tea." In general, the use of chamomile for the treatment of diseases related to CNS has not been very common in the Estonian folk medicine. However, there are some descriptions related to the use of chamomile for curing CNS diseases, and this information is confirmed by scientific studies. Numerous clinical trials have shown the sedative and hypnotic effects of chamomile, thus supporting the treatment of anxiety, depression and insomnia [21].

In our study, total 22 phenolic compounds were found in the German chamomile extracts, and of these compounds hydroxycinnamic acids were the predominant ones. The major hydroxycinnamic acids in the present dry extracts were 4.5-dicaffeoylquinic acid, 3.5-dicaffeoylquinic acid, 3.4-dicaffeoylquinic acid, chlorogenic acid, and neochlorogenic acid. In our chamomile dry extracts, however, the predominate substances were not the same as described in the literature [22,23]. Mulinacci et al. [23] reported that the extracts of chamomile flowers contain 39% cinnamic acid derivatives, such as ferulic acid and caffeic acid.

In the dry extracts of German chamomile flowers, luteolin and quercetin derivatives were found as the predominate flavonoids, and apigenin, kaempferol and isorhamnetin derivatives were present in smaller contents. The most dominant flavonoids are luteolin-7-O-glucoside and isoquercitrin. Recently, Catani et al. [22] reported that the major flavonoids present in the German chamomile raw material are apigenin, quercetin, patuletin, and luteolin at the concentrations of 16.8%, 9.9%, 6.5% and 1.9%, respectively. The results reported by Catani et al. [22] are not in line with our findings in the current study.

It should be noted that the significant amount of 3,4-dihydroxyphenylacetic acid in the extract, could be related to analgesic activity [24].

In chamomile essential oil, total nine main compounds were quantified representing more than 97% of the total oil. Table 3 shows the RI values of the principal compounds on the two columns representing different polarity and concentration ranges (>1%). a-Bisabolol oxide A [44%), B [22%), and α-bisabolone oxide A [16%) were the main compounds in the essential oil. By the chemical content, the present chamomile essential oil belongs to the chemotype rich in bisabolol oxides described in the European Pharmacopoeia [25]. Previously, we studied the essential oil of Pineapple weed [*Matricaria discoidea* DC.), which is similar to a German chamomile oil but is poor in bisabolol oxides [26].

According to the literature, the main components of German chamomile are terpenoids, such as -bisabolol and its oxide azulenes, chamazulene (1–15%) and apigenin [1,27–29]. The phenolic composition of our extracts, however, differed from the findings reported in the state-of-the literature.

We found that in preparing the dry extracts of chamomile the effective ratio of extractant to raw materials is in the range of DIR 1:16 - 1:18 (Figure 1). With this ratio, the content of extractive substances reached a "plateau" and was not significantly increased with an increase in the amount of extractant used. When extracting hydroxycinnamic acids and flavonoids, it is advisable to use the ratio of extractant to raw material in the range of DIR 1:12-1:14. Increasing the amount of the extractant does not significantly increase the output of these BASs. At the same time, it is more appropriate to extract phenolic compounds in the range of DIR 1:14-1:16. In summary, for the highest yield of phenolic compounds in the extract, the ratio of extractant to raw materials should be 1:14-1:16, and a triple extraction is recommended to be used.

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The present study demonstrates that the administration of German chamomile extracts *via* an intragastric route enhances an analgesic effect in mice in a hot-plate test. The administration of the extracts G1 and G3 resulted in a slight prolongation of the time of a discomfort occurrence. The administration of extract G1 at the dose of 50 mg/kg and 100 mg/kg extended the time period spent on the test plate with the mice (one hour after administration and before the discomfort reaction occurrence) by 38% and 43% ($p<0.05$), respectively. Interestingly, the administration of the extract G3 increased the corresponding time period only by 17% and 25%, respectively. The comparison was made to the corresponding time period observed with the control group of intact animals.

The administration of extract G2 to mice at all three doses studied and at different time points, resulted in a significant ($p<0.05$) analgesic effect in the rodents in comparison to the control group of intact animals and the group of animals that received acetaminophen. The maximum analgesia was found at 60 minutes after administration. The time of a discomfort occurrence in the animal group having the extract at the dose of 50 mg/kg and 100 mg/kg was 67% and 74% higher compared to that observed with a control group, respectively. Moreover, the analgesic activity of these extracts at the doses studied and one hour after the administration, was increased by 14% and 28%, respectively. The comparison was made to the analgesic activity found with the animal group treated with acetaminophen.

The present results obtained with a hot-plate test in mice show the analgesic activity of the German chamomile extracts studied. In the recent study reported by Chaves et al. [30], the analgesic activity of a crude chamomile fraction was investigated with a formalin test. The Authors reported that reduced nociception (by 96%) was observed upon using a 30 mg/kg dose compared to the control (10 ml/kg of saline solution), thus demonstrating analgesic properties. These findings are consistent with our results. To our best knowledge, no other studies have been published on the analgesic activity of German chamomile preparations in the state-of-the-art literature.

The vast majority of the studies published to date have reported the anti-inflammatory activity of chamomile products [1], while only little is known about their analgesic activity accompanied by anti-inflammatory activity. Lee et al. [31] studied the applicability and efficiency of German chamomile fixed oil with an atopic dermatitis animal (mice) model [31]. After the administration period of 4 weeks, there was a significant reduction in serum IgE and IgG1 levels in mice. Bhaskaran and co-workers [32] investigated the dried chamomile flower extracts and their mechanism of action in inflammatory disorders. The role of luteolin (flavonoid) in generating the anti-inflammatory effects of chamomile has also been studied and discussed [33]. Flemming et el. [34] investigated the anti-inflammatory activity of matricine from chamomile flowers *in vivo* with mice using a carrageenan-induced inflammation and air pouch models. The results showed a significant dose-dependent increase in anti-inflammatory responses in mice. The potential effects of matricin and chamazulene on inflammation were studied [34]. Some earlier studies reported also the anti-inflammatory activity of essential oil components, such as α-bisabolol, bisabolonoxid [35] and polyketides [36]. However, there is still very limited information about the analgesic activity of chamomile and its preparations.

Chamomile tea has been used for centuries for inducing calmness and for the treatment of sleep disorders [1]. It has been reported that the sedative effect is mainly due to the action of an apigenin flavonoid found in chamomile [1,37]. Apigenin acts by binding to benzodiazepine receptors present in the brain.

Thiopental sodium (barbiturate) induces efficiently sleep in both humans and rodents. A thiopental sodium-induced sleeping test is widely used for the study of the sedative-soporific activity of new active pharmaceutical ingredients [38]. We found that the German chamomile extracts (G1-G3) in rats have a sedative effect of 30.5-117.3% compared to the control group. With the rat group having the extract G1, the average duration of sleep was 140.3±6.5 min at the dose of 25 mg/kg, 201.8±4.7 min at the dose of 50 mg/kg, and 148.8±3.9 min at the dose of 100 mg/kg. These values for a sleeping time period are significantly higher (by 33.9%, 92.5% and 42.0%, respectively) compared to the corresponding time period observed with a control group ($p<0.05$). The duration of sleep was prolonged in all groups having the extract G3 by 69.6%, 30.5%, and 58.7%, respectively.

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The administration of extract G2 at a dose of 50 mg/kg resulted in the highest sedative effect in rats, and consequently, the duration of sleep increased by 117.3% compared to that observed with a control animal group. The duration of sleep was also longer than an average sleeping period in a reference group having a Valerian syrup. Reducing the extract G2 dose to 25 mg/kg led to a slight decrease in sedative activity (77.7%) in rats compared to the control group ($p<0.05$). The increase in the dose of G2 extract from 50 mg/kg to 100 mg/kg did not have any influence on the sedative activity in rats in comparison to a rodent group treated with the extract G2 at a dose of 50 mg/kg. This suggests that the administration of the extract G2 at a dose of 50 mg/kg provides a sedative action in rats with a pronounced pharmacodynamics effect.

We found that the German chamomile extracts exhibit a synergistic soporific effect with thiopental sodium. A dose-dependent decrease in locomotion was observed with mice, and the maximum effect was achieved at a dose of 30 mg/kg of the chamomile crude fraction. Mice usually demonstrate anxiolytic activity by burying noxious stuff. Several earlier studies report the anti-anxiety effects of chamomile products [30]. Moreover, chamomile and its preparations could affect fluctuations in cortisol levels associated with CNS disorders [39]. Carpenter et al. [40] reported that the elevated levels of adrenocorticotropic (ACTH) are associated with stress and anxiety. Yamamoto et al. [41] found that chamomile extracts possess neurokinin-1 receptor antagonist activity [40]. Furthermore, the inhalation of chamomile oil vapors was shown to reduce the ACTH levels caused by the stress induced by ovariectomy in rats [42]. Recently, Amsterdam et al. [43] reported that flavonoid components in chamomile modulate central neurotransmitter activity (i.e., a reduction in serotonin, dopamine and monoamine oxidase activity), elevate catecholamine production and noradrenalin activity. In addition, chamomile possesses ingredients that play an important role in CNS diseases, such as epilepsy and Alzheimer's disease. In the study carried out by Hashemi and co-workers, convulsions were induced by the administration of kainic acid [44]. To date, numerous studies have been published on the effects of chamomile products on the CNS [1]. However, only little is known about chamomile's effect on healthy users' sleep. This shows the novelty of our study.

4. Materials and Methods

4.1. Ethnomedical study

The Estonian Literary Museum is a research and development institution managing important archives of cultural history and folklore. In the ethnomedicine of Estonia, the plants from the genus Matricaria (Chamomile) have been used to relieve various health problems. The ethnomedicinal catalog entitled "Chamomile" belongs to the card index "Ethnobotany". In our study, we used these established sources of information, and a categorized electronic dataset was conducted to enable further analysis.

4.2. Plant material

German chamomile flowers (1.0 kg) were collected from the herb company MK Loodusravi OÜ field of medicinal plants located in the Päpe farm, Venevere village, Põhja-Sakala municipality, Viljandi county, Estonia [58.598228 N, 25.704950 E). The flowers were dried at 30-35 °C and stored in an airtight plastic for further studies. The identity of the raw material was established by Prof. Ain Raal, Institute of Pharmacy, University of Tartu, Tartu Estonia [25,45]. The raw material was standardized according to the European Pharmacopeia requirements [25]. The loss of drying of flowers was 6.8% [25].

4.3. Preparation of extracts

A total of 100.0 g of the dried German chamomile flowers [25] was filled with 1250.0 ml of water R and distillated in the essential oil extraction [Albrigi Luigi SRL, Stallavena, Italy) for 3 hours to get the essential oil. The content of the essential oil was 5 ml/kg in the dry raw material. After cooling, the aqueous distillated extract was separated by paper filtration from the raw material. The volume of the extract was 705 ml. The dry residue of the extract was 4.1 ± 0.4%. The distillated extract was

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evaporated to a dry extract (extract G1) by lyophilic drying in a SCANVAC COOLSAFE 55-4 Pro (LaboGene ApS, Denmark) apparatus. The yield of the dry extract G1 was 28.9%.

Total 500.0 g of the dried German chamomile flowers [25] were macerated with 3000 ml of 70% aqueous ethanol solution in an extractor at an ambient room temperature for overnight. The process was repeated five times more with 1000.0 ml of the same solvent to settle the optimal multiplicity of extraction. The first three liquid extracts were combined, then kept for sedimentation for two days, and finally filtrated. The liquid extract was then evaporated with a rotary vacuum evaporator to a thick extract, which was dried by lyophilic drying in a SCANVAC COOLSAFE 55-4 Pro (LaboGene ApS, Denmark) apparatus. The yield of the dry extract G2 was 31.9%.

The waste of a raw material (after a three-step extraction with 70% aqueous ethanol solution), was mixed with 1000.0 ml of water R and boiled for 30 min. After cooling, the aqueous extract was separated by paper filtration from the raw material and evaporated to a dry extract (extract G3) by lyophilic drying in a SCANVAC COOLSAFE 55-4 Pro (LaboGene ApS, Denmark) apparatus. The yield of the dry extract G3 was 7.5%.

4.4. Phytochemical analysis

4.4.1. Assay of main phytochemicals

The quantification of hydroxycinnamic acids, flavonoids, and total phenols in the chamomile extracts was performed with a Shimadzu UV-1800 [Shimadzu Corporation, Japan) spectrophotometer. Hydroxycinnamic acids were determined in terms of chlorogenic acid at 525 nm after reaction with hydrochloric acid, sodium nitrite and sodium molybdate [25,46,47]. Flavonoids were assayed in terms of rutin at 417 nm after the formation of the complex with aluminum chloride [7,25,48]. The content of total phenolic compounds was determined in terms of gallic acid at 270 nm [49]. For statistical validity, the experiments were performed in triplicate.

4.4.2. Gas-chromatographic analysis of essential oil

The main compounds (>1%) of chamomile essential oil were analyzed using Agilent's GC 7890a chromatograph (Santa Clara, CA, USA) with Agilent Open Lab CDS Chem Station software and FID. The analysis was conducted on two fused silica capillary columns with stationary phases DB-5 and HP-Innowax (both 30 m × 0.25 mm, Agilent). The carrier gas used was hydrogen with a split ratio 1:150 and a flow rate of 30 ml/min. The temperature program ranged from 50 to 250 °C at 2.92 °C/min, while the injector temperature was 250 °C.

The Agilent Open Lab CDS Chem Station software was used to identify the oil principal components and to compare their retention indices. The component content (%) of essential oil was determined by analyzing the mean retention time and peak area of four parallel chromatograms. We identified the components by comparing their DB-5 column retention indices to databases and literature data [9–11,37].

4.4.3. Identification of phenolic compounds by UPLC-MS/MS

Determination of phenolic compounds in German chamomile flowers was carried out with an UPLC-MS/MS system. Chromatographic separation was performed using Acquity H-class UPLC system [Waters, USA) equipped with YMC Triart C18 [100 × 2.0 mm 1.9 µm) column. The constant temperature of the column was 40 °C during analysis. The flow rate of a mobile phase was 0.5 ml/min. Aqueous solution of formic acid (0.1%) was used as a solvent A, and MS-grade acetonitrile was used as a solvent B. The following linear gradient was applied: Solvent A from 0 to 1 min isocratic conditions at 95%, 1 to 5 min. linear decrease to 70%, 5 to 7 min. to 50%, 7.5 to 8 min. wash column with 100% solvent B, and 8.1 to 10 min. equilibrate column with initial conditions. Mass spectrometric analysis was carried out with a triple quadrupole tandem mass spectrometer [Xevo, Waters, USA). Negative electrospray ionization [ESI) was performed to acquire MS/MS data. The settings for MS/MS analysis were as follows: voltage of capillary tip set at negative 2 kV, nitrogen gas heated to 400 °C was flowing at 700 l/h, curtain gas flow set at 20 l/h, ion source temperature was set at 150 °C.

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Identification of phenolic compounds in German chamomile flowers were established by comparing their retention times and MS/MS spectral data with those of commercial reference substances. The quantitative determination of phenolic compounds was performed with a standard dilution method and linear regression fit models for phenolic compounds [50].

4.5. Pharmacological study

All pharmacological studies (analgesic and soporific activity) were carried out in compliance with the rules of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" [Strasbourg, 1986); Directive 2010/63/EU of the European Parliament and of the Council of the European Union [2010) on protection of animals used for scientific purposes; the Order of the Ministry of Health of Ukraine No. 944 "On Approval of the Procedure for Preclinical Study of Medicinal Products and Examination of Materials for Preclinical Study of Medicinal Products" [2009); the Law of Ukraine No. 3447-IV "On the protection of animals from cruel treatment" [2006) [51–55]. The research was approved by the Bioethics Commission of the National University of Pharmacy [protocol №4 from 03.10.2023).

For the pharmacological study, the rodents (mice and rats) were kept on a standard diet and conditions in the vivarium of the National University of Pharmacy [Kharkiv, Ukraine).

4.5.1. Analgesic activity

The analgesic activity of chamomile extracts (G1, G2, G3) and the reference drug acetaminophen (Paracetamol-Zdorovye, capsules 500 mg, Pharmaceutical company «Zdorovye», Kharkiv, Ukraine) was studied with mice weighing 20-40 g.

The animals were kept without food for 2 hours before the test. The groups of animals were formed by the method of randomization. The period of quarantine and acclimatization lasted for 14 days. The mice were divided into 11 groups (6 mice in each group): Group 1 – intact animals, who received a 0.9% solution of NaCl in a dose of 1 ml per 100 g of body weight; Group 2 – animals received G1 extract at a dose of 25 mg/kg; Group 3 – animals received the extract G1 at a dose of 50 mg/kg; Group 4 – animals received the extract G1 at a dose of 100 mg/kg; Group 5 – animals received the extract G2 at a dose of 25 mg/kg; Group 6 – animals received the extract G2 at a dose of 50 mg/kg; Group 7 – animals received the extract G2 at a dose of 100 mg/kg; Group 8 – received the extract G3 at a dose of 25 mg/kg; Group 9 – animals received the extract G3 at a dose of 50 mg/kg; Group 10 – animals received the extract G3 at a dose of 100 mg/kg; Group 11 (control group CG) – animals received acetaminophen at a dose of 50 mg/kg.

The chamomile extracts studied were administered intragastrically at a dose of 25 mg/kg, 50 mg/kg and 100 mg/kg in the form of an aqueous suspension 30 minutes before placing the animals on the equipment. The reference drug, acetaminophen, was administered at a dose of 50 mg/kg as a solution.

After the administration of the extract or reference drug the animal was carefully placed on a hot plate (55°C) in 30 minutes. The indicator of pain sensitivity was the duration of the animal's stay (in seconds) on the hot plate before the onset of protective reflexes (licking of limbs, rebound). The mice were observed for 0.5, 1, 2, 3 and 4 hours. The criterion of the analgesic effect was a significant increase in the latent period of response after the administration of the sample (extract or drug) compared to the control. To prevent thermal damage during the experiment, the time of exposure to animals in the test on a hot plate did not exceed 60 seconds. On the "hot plate" model, the analgesic activity was calculated according to the following equation:

$$AA = \frac{\mathrm{Te}-\mathrm{Tc}}{\mathrm{Tc}} \times 100\%;$$

where AA – is the analgesic activity, %;

Te – is the difference in the latent period of the corresponding response in the group of experimental animals before and after administration of a potential analgesic;

 doi:10.20944/preprints202312.0294.v1

Tc – is the difference in the latent period of the corresponding response in the group of control animals before and after administration of the solvent.

Statistical data analysis was performed using parametric methods of statistics by Student t-test. The level of statistical significance of differences was $p<0.05$.

4.5.2. Soporific activity

The soporific activity of chamomile extracts (G1, G2, G3), sodium thiopental lyophilisate (for injection solution, PLC "Kiivmedpreparat", Kyiv, Ukraine), and "Valerian syrup AN NATUREL" syrup (LLC Beauty and Health, Kharkiv, Ukraine) was investigated with white rats weighing 190-280 g.

The animals were divided into 12 groups (6 rats in each group): Group 1 – intact animals; Group 2 – animals received G1 extract at a dose of 25 mg/kg; Group 3 – animals received the extract G1 at a dose of 50 mg/kg; Group 4 – animals received the extract G1 at a dose of 100 mg/kg; Group 5 – animals received the extract G2 at a dose of 25 mg/kg; Group 6 – animals received the extract G2 at a dose of 50 mg/kg; Group 7 – animals received the extract G2 at a dose of 100 mg/kg; Group 8 – animals received the extract G3 at a dose of 25 mg/kg; Group 9 – animals received the extract G3 at a dose of 50 mg/kg; Group 10 – animals received the extract G3 at a dose of 100 mg/kg; Group 11 (control group CG1) – animals received the reference drug sodium thiopental at a dose of 40 mg/kg, and Group 12 (control group CG2) (Valerian) – animals received Valerian syrup at a dose of 2.14 mg/kg. The duration of sleep was determined by the time period while the rats were in a lateral position.

4.6. Statistical analysis

The mean and standard deviation (SD) were calculated according to the Monograph "Statistical Analysis of the Results of a Chemical Experiment" of the State Pharmacopoeia of Ukraine [25,56]. The average value was established on the basis of at least three (3) measurements in the phytochemical study and on basis of six (6) measurements in the pharmacological study. The values of the confidence interval were calculated using a Student's criterion limit. The data are presented as the mean ± SD [25,57].

5. Conclusions

In the present study, two novel methods for preparing essential oil and dry extracts from the tincture and aqueous extracts of German chamomile flowers were introduced. Total 22 phenolic compounds were identified and quantified in the dry extracts of German chamomile flowers. Total 9 terpenoids were identified and quantified in the essential oil of German chamomile flowers. The content of the main phenolic compounds was successfully determined by means of spectrophotometry. The dry extracts of German chamomile flowers show an analgesic activity in a mice model and improve the sleep in a rat model. The dry extract prepared from a 70% aqueous ethanol solution show the highest analgesic and soporific efficiency in rodents. Our study provides also evidence about the sedative use of chamomiles in the Estonian folk medicine.

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