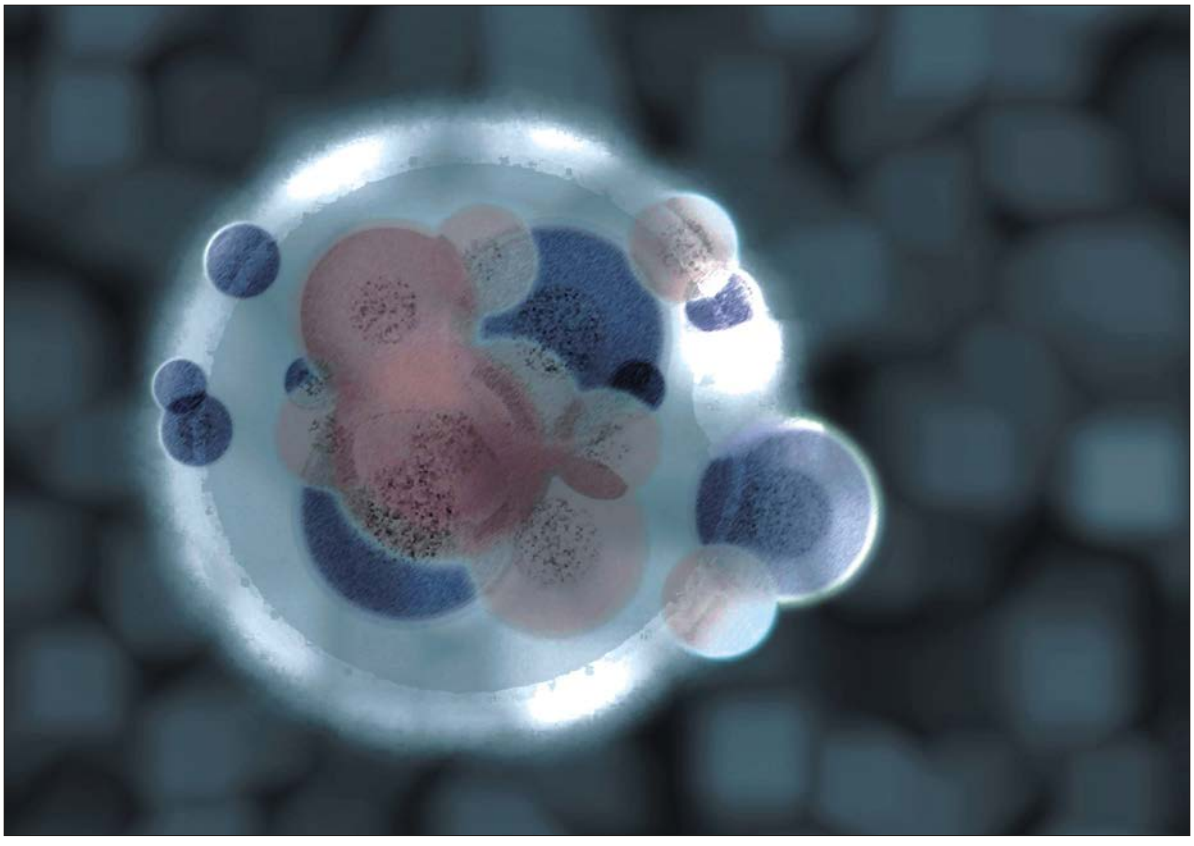

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Research Article
Callogenesis and Analgesic Evaluation of Adult Plant Extracts and Callus in *Teucrium polium* L. Subsp. *geyrii* Maire

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Abstract

Background and Objective: *Teucrium polium* L. subsp. *geyrii* (*T. polium* L. subsp. *geyrii*) Maire is a saharo-endemic subspecies commonly used in Algerian traditional medicine for its therapeutic virtues. The analgesic effects of the aqueous infusion extract from aerial part of *T. polium* L. subsp. *geyrii* Maire collected *in situ* and of its homologue prepared from the callus infusion as well as the various phenolic extracts (diethyl ether extract, n-butanol extract and hydroalcoholic extract) were investigated and reported for the first time. **Materials and Methods:** Eight media were used for the induction of callogenesis from apical buds, nodals, leaves and flowers of *T. polium* L. subsp. *geyrii* Maire. High-Performance Liquid Chromatography (HPLC) analysis was used to identify phenolic compounds present in diethyl ether extract and callus infusion. The study of the analgesic activity of different plant extracts was conducted using acetic acid induced writhing method on mice. **Results:** The media M1, M2 and M7 were effective in inducing callus and gave the highest rates of callus. Apical buds and flowers were the most reactive organs. Aqueous infusion extract and the phenolic extracts showed excellent analgesic effects even better than the reference drug. The oral administrations of the callus infusion significantly ($p<0.001$) decreased the number of writhing induced by acetic acid and showed the highest level of protection compared to all the extracts tested and to the reference with 88.59%. HPLC profile obtained from the phenolic extracts of *T. polium* L. subsp. *geyrii* Maire and its callus infusion showed the presence of quercetin and other phenolic compounds, which may explain the high analgesic effect of this sub-species. **Conclusion:** The *T. polium* L. subsp. *geyrii* Maire has a significant analgesic effect and confirms its traditional use by the Targuie population.

Key words: *Teucrium polium* L. subsp. *geyrii* Maire, polyphenols, callus, chromatography analysis, analgesic effect

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Medicinal plants have been used for centuries to treat many ailments as these plants are a source of molecules with therapeutic potential. The interest in herbal medicine which represents an important tool for the identification of novel drug leads to increase greatly[1]. Thus, study of plant species that traditionally have been used as pain killers may be seen as a logical research strategy, in search for new drugs with analgesic effects[2].

As the demand for medicinal plants is growing at a very fast pace, consequently some of them are increasingly being threatened in their natural habitats[3]. Therefore, in search of alternatives for production of desirable medicinal compounds from plants, biotechnological approaches, specifically plant tissue cultures (callus culture, cell suspension culture and/or organ culture) are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites[4]. Cell culture technologies were introduced as a possible tool for studying and producing plant secondary metabolites as *in vitro* regeneration holds tremendous potential for the production of high quality plant based medicines[5].

The genus *Teucrium* L., with about 300 species, is a large and polymorphic genus distributed in Europe, North Africa and temperate parts of Asia but mainly in the Mediterranean region[6]. *Teucrium polium* is a wild growing flowering plant belonging to the Lamiaceae family. The sub-species *T. polium* L. subsp. *geyrii* Maire is an Algerian endemic spontaneous species known by the Touareg as "Takmazzut" which grows mainly in stony beds of wadis and in rocks at altitudes between 1200 and 2600 m[7].

Ethnobotanically, this plant is used for preparation of tea and as a spice plant. *In vivo* and *in vitro* pharmacological studies reported the anti-inflammatory, antipyretic, hypoglycemic, anticancer, antioxidant, antifungal and antibacterial activities of *T. polium*[8-13]. It also has a beneficial effect on wounds[14]. These effects may be due to the presence of bioactive molecules particularly polyphenolic compounds that many studies reported their *in vitro* and *in vivo* properties[15-17].

In Algeria, *T. polium* L. subsp. *geyrii* Maire as well as many medicinal plants are threatened and facing disappearance at an alarming rate. This might be due to uncontrolled collection of plant materials, the climatic changes and the destruction of their natural habitats. This requires *in vitro* conservation of this traditional medicinal plant, chemical analysis and pharmacological studies. In fact, the present study describes a protocol that has been developed for the induction of callus obtained from apical buds, nodals, leaves and flowers. Furthermore, the present study aimed to determine the analgesic activity of callus obtained on the Swiss albino mice as well as its comparison with the aqueous infusion and the phenolic extracts obtained from aerial parts of *T. polium* L. subsp. *geyrii* Maire. In addition, an attempt to determine the chemical constituents responsible for this analgesic activity was made.

MATERIALS AND METHODS

Chemicals and reagents: Benomyl, calcium hypochlorite, HCl, ether, diethyl ether, ethanol, methanol, n-butanol, acetic acid and Murashige and Skoog basal medium were obtained from Sigma Aldrich (America). The plant growth hormones: 6-Benzylaminopurine and α-naphthaleneacetic acid were purchased from Duchefa Biochemie, (Netherlands), kinetin and 2,4-Dichlorophenoxyacetic acid were purchased from Alfa Aesar GmbH and Co KG, (Germany). Aspirin was purchased from Saidal (Algeria) and all other chemicals and reagents were of analytical grade.

Plant material collection: *T. polium* L. subsp. *geyrii* Maire was collected at the flowering stage in April, 2013, in Ilamen station which is located in Tamanrasset (Algerian central Sahara) (longitude 5° 29' 30'', latitude 23°14' 26,9'', Altitude, 2055 m). Plant samples were identified by the National Institute of the Forestry Research (INRF), Tamanrasset, Algeria and a voucher specimen (MP-19-4-13) was deposited at the herbarium of the Research Laboratory on Arid Zones (LRZA, USTHB), Algiers, Algeria.

A part of the fresh aerial parts of the plants were used for the callus induction and the rest of the aerial parts were dried in the shade at room temperature and then preserved in paper bags in order to avoid possible fungal contamination. Plants were cut into small pieces, then crushed into a fine powder by a commercial mechanical grinder (Krups 75) and preserved at 4°C for further analysis. The infusion of *T. polium* L. subsp. *geyrii* Maire aerial parts and callus was prepared according to the traditional method, the fine powder of the plant and the lyophilized callus were dissolved in boiling normal saline water, the hot infusion was then left to reach room temperature and filtered. The filtrate was used for biological tests on animals at different doses.

Animals: Swiss albino mice (*Mus musculus*) weighing 20±5 g, were kindly provided by the Laboratory of Microbiology of the Centre of Research and Development (CRD-Saidal), Algeria. The animals were fed pellet diet from the National Office of Animal Feed, Bejaia (ONAB) and water *ad libitum*. The standardized laboratory conditions of temperature and light (24±1°C and 12 h light/dark cycle) with a 50% of humidity were also kept and maintained. All experimental procedures were approved by the University Animal Experimentation Ethics Committee (approval ref No. 162/2011/8) and following the guidelines prescribed in Guide for the Care and Use of Laboratory Animals.

Callus induction: Young shoots (apical buds, nodals, leaves and flowers) of *T. polium* L. subsp. *geyrii* Maire were used as explants source for callus induction. For surface disinfection, the shoots were first washed with running tap water for about 15 min, then immersed in 70% (v/v) ethanol for 1 min and by benomyl (3 g L^{-1}) for 30 min and finally with calcium hypochlorite (35 g L^{-1}) for 15 min followed by several washes with sterilized distilled water in order to remove excess of the chemicals. After drying on filter paper, these shoots were fragmented into explants (1-1.5 cm in length) and then placed on sterilized callus induction solid basal Murashige and Skoog[18] (MS) medium supplemented with various concentrations and combinations of plant growth regulators (PGRs), namely 6-Benzylaminopurine (BAP), kinetin (KIN), α-Naphthalene acetic acid (NAA), 2,4-Dichlorophenoxy acetic acid (2,4-D) as follow:

- Medium 1 [M1: KIN (1 mg L^{-1}), NAA (1 mg L^{-1})]
- Medium 2 [M2: KIN (1 mg L^{-1}), 2,4-D (1 mg L^{-1})]
- Medium 3 [M3: BAP (2 mg L^{-1}), NAA (2 mg L^{-1})]
- Medium 4 [M4: BAP (2 mg L^{-1}), 2,4-D (2 mg L^{-1})]
- Medium 5 [M5: BAP (1mg L^{-1}), AIB (1mg L^{-1})]
- Medium 6 [M6: BAP (2 mg L^{-1}), AIB (2mg L^{-1})]
- Medium 7 [M7: KIN (1 mg L^{-1}), AIB (1 mg L^{-1})]
- Medium 8 [M8: KIN (2 mg L^{-1}), AIB (2 mg L^{-1})]

The 30 g L^{-1} of sucrose as carbon source and 8 g L^{-1} of agar-agar (Prolabo VWR chemicals) as gelling agent were added to all the media. The pH of all the media was adjusted to 5.7 before their sterilization at 120°C and a pressure of 100 kPa in the autoclave. All culture was incubated in darkness at a temperature of 28±2°C.

Phenolic compounds extraction methods: In order to extract the maximum of polyphenols present in the studied plant, several extraction methods on the powder of *T. polium* L. subsp. *geyrii* Maire adult mother plant were carried out. The extraction of flavonoid aglycones was carried out based on the procedure described by Lebreton *et al.*[19] from the initial method of Bath-Smith[20]. It consisted of a hot hydrochloric acid hydrolysis HCl (2N, 80 mL) of 1g of *T. polium* L. subsp. *geyrii* Maire aerial parts powder, causing the release of phenolic acids and flavones-flavonols by using diethyl ether (ExDi-Eth). From the remaining aqueous phase, the anthocyanidins and C-glycosides by n-butanol (ExButOH) were extracted. The extraction of flavonic glycosides was assessed using the method described by Harborne[21]. Flavonic glycosides (C-glycosides and O-glycosides) extraction was done by cold maceration method. One gram of *T. polium* L. subsp. *geyrii* Maire powder was macerated in 100 mL of ethanol (70%) for 48 h. The hydroalcoholic extract (ExHydOH) was then shacked, filtered and evaporated in rotating evaporator under reduced pressure until dryness. The dry residue was recovered in 100 mL boiling distilled water. The resultant aqueous solution, then, was extracted by 50 mL of n-butanol. All the extracts (diethyl ether extract, n-butanol extract and hydroalcoholic extract) were dried by evaporation and dissolved in 3 mL pure methanol then kept in a refrigerator at +4°C for further analysis.

HPLC analysis: High-Performance Liquid Chromatography (HPLC) was performed using a Waters Alliance 2695 chromatography system and UV detector with bars of diodes surveyor. Chromatographic runs were performed using C18 column (20×4.6 mm, 5 µm). The chromatographic data system was controlled by the Empower Software from Waters. The extracts were analyzed using solvent system B (methanol) in solvent system A (0.1% phosphoric acid in water) with the following gradient: 1-10 min (30% B), 1-20 min (30-40% B) and 20-60 min (40-100% B). The solvent flow rate was 1 mL min^{-1} and detection was performed at 260 and 365 nm.

Analgesic activity: The analgesic activity was evaluated using acetic acid induced writhing method in mice following the method of Das *et al.*[22]. The animals were fasted for 19 h prior to experiments. They were divided into 12 groups containing 6 mice each administered orally with either vehicle control [normal saline water, 0.5 mL/20 g body weight (b.wt.)], aspirin (500 mg kg^{-1} b.wt.), infusion of *T. polium* L. subsp. *geyrii*

Maire adult mother (300, 500 and 1000 mg kg^{-1} b.wt.), callus infusion (500 mg kg^{-1} b.wt.), diethyl ether extract (300 and 500 mg kg^{-1} b.wt.), n-butanol extract (300 and 500 mg kg^{-1} b.wt.) or hydroalcoholic extract (300 and 500 mg kg^{-1} b.wt.). All extracts and control were administered orally 30 min before intraperitoneal injection of acetic acid (0.6%, 10 mL kg^{-1} b.wt.). After the injection of acetic acid, the animals were placed in transparent plastic cages and each mouse was observed individually for counting the number of writhing, they made in 10 min beginning just 5 min after the intraperitoneal administration of acetic acid solution. The inhibition percentage (protection percentage) was calculated by the following formula:

$$\text{Protection percentage} = \frac{\text{Number of writhing of control group-number of writhing of treated group}}{\text{Number of cramps of control group}} \times 100$$

Statistical analysis: The data were presented as Mean±SD. Significance between control and treated groups was tested by one-way ANOVA followed by Tukey t-test. The $p<0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Callus induction: Apical buds, nodals, leaves and flowers of *T. polium* L. subsp. *geyrii* Maire were used to induce callus on eight chosen media (M1, M2, M3, M4, M5, M6, M7 and M8).

These different explants responded differently to various concentrations and combinations of PGRs. Callus appeared from different explants on all culture media at about 1 month after culture. The callus induction rate and type varied depending on explants used as well as the culture media employed.

As shown in Table 1, callus formation from different explants was observed on almost all media except the nodal explants cultured on M5 and M6. The maximum percentage (99%) of callus induction was achieved from the apical buds explants cultured on the M1 medium. Among the four explants tested, apical buds and flowers produced the maximum response for the induction of callus compared with nodals and leaves explants. In the present study, explants such as flowers cultured on M1 and M2 media showed a better response for callus induction than those on others (M3, M4, M5, M6, M7 and M8) with 91 and 93%, respectively. In the case of leaves explants, the best induction percentage of 65.01% was recorded in M2 medium follow by 38.1% in M1 medium while M5 medium showed the lowest response for callus induction with 12.1%. For nodals explants, maximum callus induction (40.2%) was observed in M1 medium while the nodals cultured on M5 and M6 did not show any sufficient callus induction potential (0%). The minimum callus induction percentages were recorded in M3 and M4 for nodals explants (1.7 and 10.3%, respectively).

Callus can probably be obtained in all plant species provided that the appropriate explant types, culture media composition and cultural environmental conditions are employed[23]. It has the potential to differentiate into a plant by a direct or indirect way and good quality callus is of great value for research and improvement in plants[24]. In this paper, the response of *T. polium* L. subsp. *geyrii* Maire explants to different auxin/cytokinin combinations was investigated. As a result, the responses of the callus to all adopted media were very satisfying. Several combinations of auxins (NAA, 2,4-D or AIB) and cytokinins (BAP or KIN) efficiently induced callogenesis where the (1 mg L^{-1} KIN+1 mg L^{-1} NAA) combination was the best for callus induction followed by the (1 mg L^{-1} KIN+1 mg L^{-1} AIB) combination for the apical buds explants and the (1 mg L^{-1} KIN+1 mg L^{-1} 2,4-D) combination for the flowers explants. Using leaves and nodals as explants, a minimum response in callus induction was found. The (BAP+AIB) combination at different concentrations (1 and 2 mg L^{-1}) did not induce any callus formation for the nodals explants, which is mainly due to

Table 1: Callus induction percentage from the different explants (apical buds, nodals, leaves and flowers) of *T. polium* L. subsp. *geyrii* Maire

	Explants of *T. polium* L. subsp. *geyrii* Maire (%)			
Medium	Apical buds	Nodals	Leaves	Flowers
M1	99.3 ± 1.1^{a}	$40.2\pm5^{f,g,h,i}$	$38.10\pm2.3^{g,h,i,j}$	$91.60\pm3.4^{a,b,c,d}$
M2	$79.7\pm0.5^{a,b,c,d}$	$19.3\pm1.1^{h,i,j,k,l}$	$65.01\pm7.3^{c,d,e,f,g}$	$93.00\pm6.7^{a,b,c}$
M3	$89.7\pm1.5^{a,b,c,d}$	$1.7\pm2.9^{k,l}$	$29.60\pm1.5^{h,i,j,k}$	$79.10\pm1.2^{a,b,c,d}$
M4	$83.1\pm3.5^{a,b,c,d}$	$10.3\pm1.8^{j,k,l}$	$22.40\pm2.0^{h,i,j,k,l}$	$67.90\pm3.1^{a,c,d,e,f}$
M5	$71.3\pm3.5^{a,b,c,d,e}$	0.0 ± 0.00^{l}	$12.10\pm2.8^{i,j,k,l}$	$63.05\pm3.8^{d,f,e,g}$
M6	$84.7\pm5.0^{a,b,c,d}$	0.0 ± 0.00^{l}	$15.20\pm0.1^{i,j,k,l}$	$46.30\pm4.9^{e,f,g,h}$
M7	$98.3\pm2.8^{a,b}$	$26.8\pm2.78^{h,i,j,k,l}$	$32.90\pm2.5^{h,i,j}$	$69.80\pm3.3^{b,c,d,e}$
M8	$62.8\pm0.6^{d,e,f,g}$	$18.8\pm1.07^{h,i,j,k,l}$	$28.03\pm2.1^{h,i,j,k,l}$	$73.80\pm4.3^{a,b,c,d,e}$

Values represent the Mean±SD. Values within the same column followed by the same letter are not significantly different according to the least significant difference at $p<0.05$ (Tukey test), Each treatment consisted of three replications. All data were collected 4 weeks after inoculated into the medium supplemented with PGRs

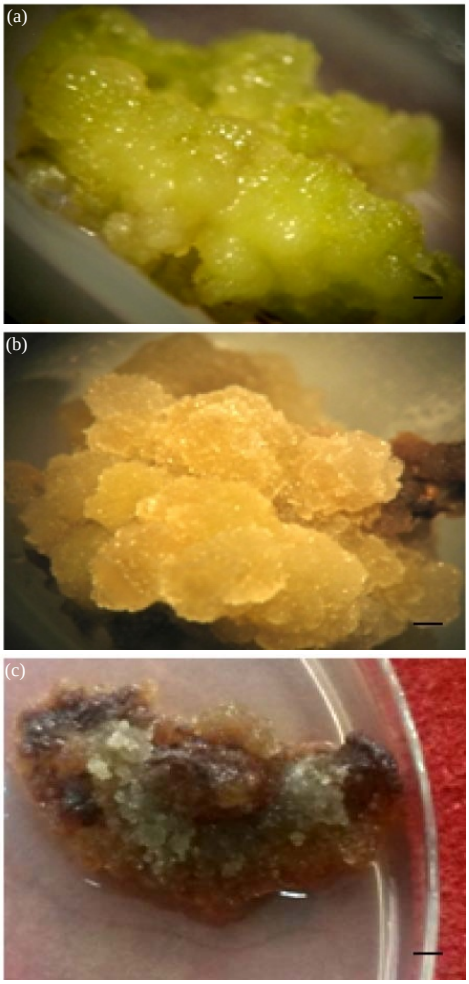

Fig. 1(a-c): Different types of *T. polium* L. subsp. *geyrii* Maire callus (a) Green nodular callus, (b) Yellow friable callus and (c) Brown and black friable callus
Scale bar = 0.5 cm

the incapacity of cells to differentiate and to express their totipotent. Similar results were obtained with the explants of *Triticum turgidum* and *Triticum aestivum*[25,26].

There are many factors affecting callus formation and regeneration. Auxin and cytokinin play fairly important roles in many aspects of plant growth and development. Understanding the auxin and cytokinin interaction in the regulation of plant development and organogenesis has advanced considerably over more than a half-century but surprisingly little is known about how they induce callus at the molecular level[27]. Many early experiments have revealed the essential roles of both hormones in the cell proliferation and new organ regeneration. It is amazing that the decision of cell fate in specific tissues depends on the ratio between auxin and cytokinin, which maintains the cell proliferation or

Table 2: Effect of the *T. polium* L. subsp. *geyrii* Maire extracts on acetic acid induced writhing in mice

Groups	Dose (mg kg^{-1} b.wt.)	Number of writhing
Control	-	57.00±1.70***
Aspirin	500	31.00±1.80$
Plant infusion	300	21.00±2.10***$
	500	15.33±2.06***$
	1000	11.83±1.90***$
Callus infusion	500	6.50±1.80***$
ExDi-Eth	300	12.50±1.80***$
	500	7.80±1.40***$
ExButOH	300	13.60±1.60***$
	500	8.83±3.43***$
ExHydOH	300	11.50±2.10***$
	500	9.00±2.20***$

Data represent Mean±SD, (n = 6), ***p<0.001 significant compared to control, $p<0.05 significant compared to aspirin, ***$Significant compared to control and aspirin (one way ANOVA followed by Tukey's test)

stimulates cell differentiation to form new organs[28,29]. The auxin combined with the cytokinin action was shown in many species, *Carica candamarcensis*, *Cicer arietinum* L., *Atriplex halimus*, *Mentha viridis* L., *Solanum laciniatum* Ait, *Vigna subterranea*, *Ocimum canum* and *Quercus suber* L.[28-35].

The calli obtained are of different colors (Fig. 1). In terms of consistency, two types of callus have been obtained, some have nodular nature and others have friable with high brittleness potential, high hydration and a significant cell division capacity after several subcultures. This friable brown callus is analyzed and tested for their analgesic activity as it has been assumed that these calli are the richest in polyphenols.

HPLC analysis: Individual phenolic compounds of *T. polium* L. subsp. *geyrii* Maire were identified by HPLC. The chromatographic separations of polyphenols in the diethyl ether extract of the aerial parts and the callus infusion of *T. polium* L. subsp. *geyrii* Maire are shown in Fig. 2 and 3, respectively. The results indicated that diethyl ether extract of the aerial parts contained 3,4,5-Trimethoxybenzoic acid, luteolin, quercetin, kaempferol, apigenin, isorhamnetin and ferulic acid whereas the callus infusion contained gallic acid, 3,4,5-Trimethoxybenzoic acid, luteolin, quercetin, kaempferol, apigenin, isorhamnetin, syringic acid, vanillic acid, protocatechuic acid and orcinol.

Analgesic activity: Analgesic activity of *T. polium* L. subsp. *geyrii* Maire aerial parts and callus infusion was evaluated using the acetic acid induced writhing method. Results showed that all extracts caused in dose-dependent manner a highly significant (p<0.001) decrease in the number of writhes at different doses compared with the control (Table 2).

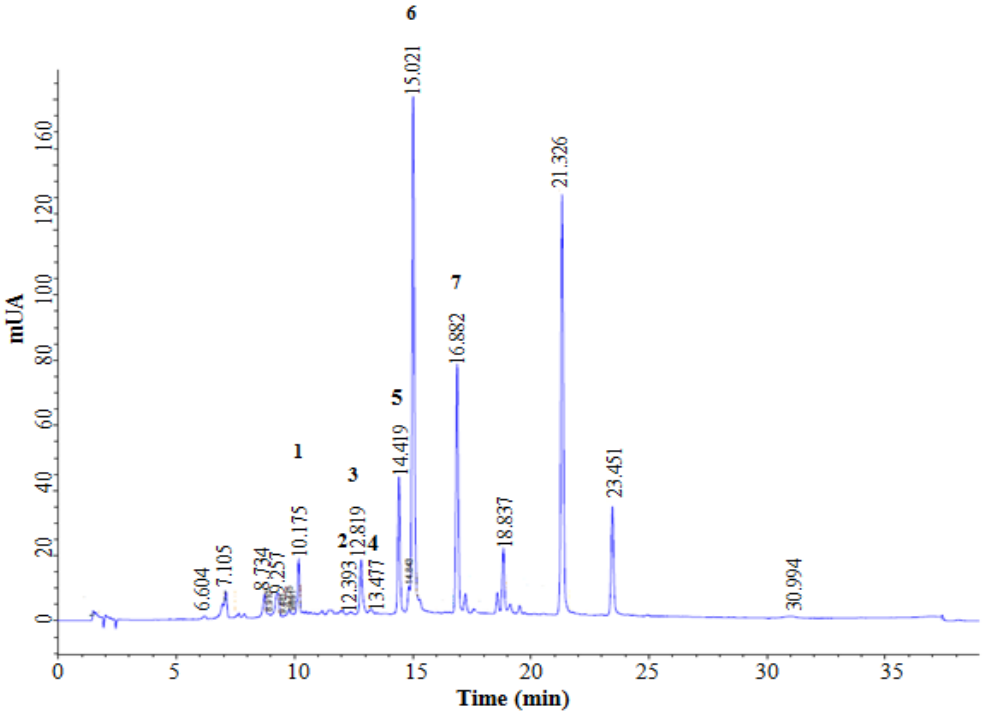

Fig. 2: HPLC chromatogram of diethyl ether extract of *T. polium* L. subsp. *geyrii* Maire aerial parts at 365 nm
(Peaks; 1: 3,4,5-Trimethoxybenzoic acid, 2: Luteolin, 3: Quercetin, 4: Kaempferol, 5: Apigenin, 6: Isorhamnetin, 7: Ferulic acid)

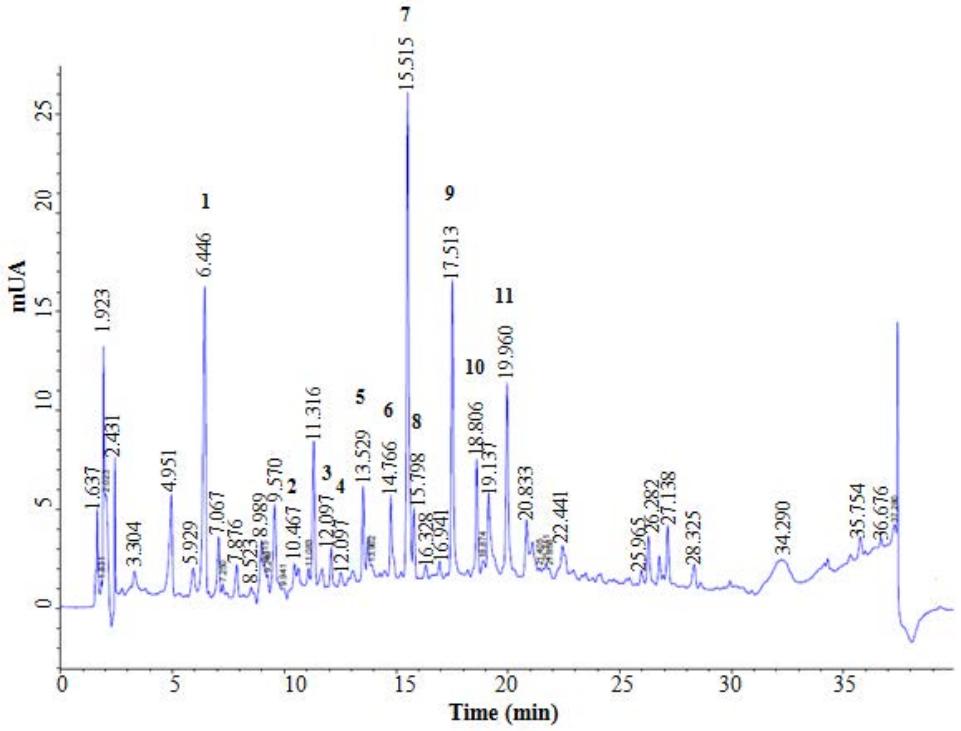

Fig. 3: HPLC chromatogram of callus infusion of *T. polium* L. subsp. *geyrii* Maire at 260 nm
(Peaks; 1: Gallic acid, 2: 3,4,5-Trimethoxybenzoic acid, 3: Luteolin, 4: Quercetin, 5: Kaempferol, 6: Apigenin, 7: Isorhamnetin, 8: Syringic acid, 9: Vanillic acid, 10: Protocatechuic acid, 11: Orcinol)

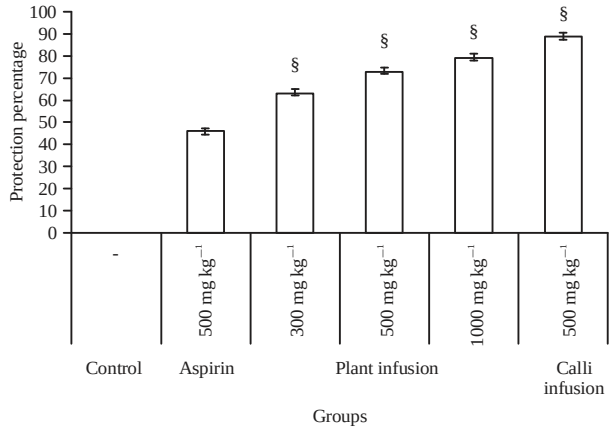

Fig. 4: Analgesic activity of *T. polium* L. subsp. *geyrii* Maire aerial parts and callus infusion using acetic acid induced writhing in mice

Data represent Mean±SD, (n = 6), §p<0.05 significant compared to aspirin (one way ANOVA followed by Tukey's test)

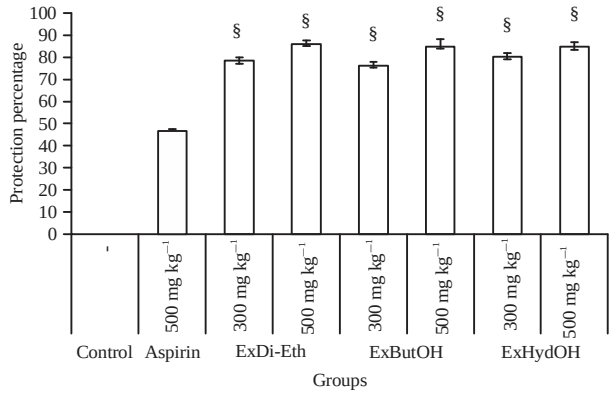

Fig. 5: Analgesic activity of *T. polium* L. subsp. *geyrii* Maire polyphenolic extracts using acetic acid induced writhing method in mice

Data represent Mean±SD, (n = 6), §p<0.05 significant compared to aspirin (one way ANOVA followed by Tukey's test)

The oral administration of aspirin as a reference drug at a dose of 500 mg kg^{-1} b.wt., significantly prevented ($p<0.001$) the appearance of contortions with 45.6% compared to the control. The analgesic effect of the callus infusion (88.59%) at the dose of 500 mg kg^{-1} b.wt. was greater than the plant infusion at all doses tested (300, 500 and 1000 mg kg^{-1} b.wt.) with an inhibition of writhing of 63.15, 73.10 and 79.24%, respectively (Fig. 4).

The hydroalcoholic extract (ExHydOH) just like the n-butanol extract (ExButOH) both at the doses of 500 mg kg^{-1} b.wt., significantly ($p<0.001$) inhibited the writhing response by 84% when compared to the control group, while the diethyl ether extract (ExDi-Eth) at the same dose significantly ($p<0.001$) had a better analgesic activity (86% inhibition) (Fig. 5).

Acetic acid, the peripheral analgesic agent, causes inflammatory pain by inducing abdominal constrictions and stretching of hind limbs. This response is believed to be mediated by the prostaglandin pathways[36]. According to the results, analgesic effect of *T. polium* L. subsp. *geyrii* Maire extracts was significantly more important than the reference drug (aspirin). It is possible that the presence of analgesic compounds in the phytochemical content of this sub-species might influence the prostaglandin pathways.

The callus induction opens new perspectives in order to make use of the potential biological activities of its infusion without harvesting the plant itself. Since the active compounds found are polyphenols that have been proved to have pharmacological properties, further studies on the structure-activity relationship will correlate the results of biological tests to specific structural features.

CONCLUSION

The obtained results showed that all extracts tested have a dose dependent analgesic action and the aqueous extract of callus had the most effective analgesic activity compared to the adult plant. It can be concluded that the use of biotechnological approaches can be of interest in the conservation of rare and endangered medicinal plants.

SIGNIFICANCE STATEMENT

This research is a first contribution to the study of the phytochemical conformity between the adult plant and the *in vitro* callus obtained from *T. polium* L. subsp. *geyrii* Maire. This study shows the possible callus induction of the medicinal plant *Teucrium polium* L. subsp. *geyrii* Maire using various plant growth regulators in one hand and in another hand, the richness of the callus infusion as well as the aerial parts infusion of the studied plant with phenolic compounds that could be responsible for the analgesic effect attributed to this plant. This research can help scientists as well as plant breeders to produce the plant and to fully benefice from their therapeutic compounds.

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