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Original Research Article

Phytochemical Characterization, Acute Toxicity and Analgesic Activity of *in vitro* Regenerated Plantlets Extract of *Teucrium polium* L. subsp. *geyrii* Maire in Mice

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ABSTRACT

This study produced *in vitro* regenerated plantlets (TPRP) from calli obtained from different explants of *Teucrium polium* L. subsp. *geyrii* Maire. The phenolic compounds from the mother plant (TPMP), callus (TPC), and TPRP were extracted, and their phytochemical variations were examined. The toxicity of the TPRP was assessed in accordance with the Organisation for Economic Co-operation and Development (OECD) Test Guideline 423. The analgesic effect was determined using the acetic acid-induced writhing test. The results indicated that transferring calli to the regeneration medium enabled the formation of new shoots on all the calli derived from explants of apical buds. Culture media enriched with 1.5 and 2 mg/L BAP yielded the best regeneration responses, though the differences were not statistically significant ($P > 0.05$). High-Performance Liquid Chromatography (HPLC) analysis of flavonoid aglycone extracts from TPMP, TPC, and TPRP showed no significant qualitative difference. However, TPMP contained markedly higher quantitative levels. TPC extracts showed no signs of toxicity, even at a dose of 2 g/kg. The results of the analgesic activity implied that the diethyl ether extract of TPRP was more effective than the hydro-alcoholic extract. The highest protection (90%) was observed with a dose of 500 mg/kg. These findings demonstrate the *in vitro* regeneration of *T. polium* L. subsp. *geyrii* and highlight the bioactive potential of regenerated plantlets, particularly their analgesic properties. The absence of toxicity and significant phytochemical composition suggests that TPRP could serve as a valuable source for therapeutic applications, warranting further pharmacological investigation.

Keywords: *Teucrium polium*, regeneration, analgesic activity, HPLC, toxicity.

Introduction

Contemporary biotechnology techniques offer significant potential for producing superior plant-based pharmaceuticals. They enable large-scale propagation of disease-free plants, faster cloning processes, and the preservation of desirable genotypes in a short period. Strong cell culture techniques have been developed to produce important secondary metabolites with pharmacological relevance.[1] Moreover, these methods have become increasingly valuable tools for both scientific research and commercial applications in recent years.[2] The micropropagating of woody plants is known as somatic embryogenesis, which can be achieved either directly or indirectly by using somatic cells.[3] The explants respond to endogenous stimuli during cell differentiation, which generates the induction of a signaling response leading to modification of the cell gene program. Somatic embryogenesis offers various scientific and biological advantages, such as the potential to improve plants of commercial importance.[4]

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In order to screen and investigate wild plants used in traditional medicine, especially those of African origin, we undertook this study on *T. polium* L. subsp. *geyrii*Maire, an endemic species found in Tamanrasset province, a mountainous region of the Algerian central Sahara. This plant, known to local people (Tuareg) as “Takmazzut”, is highly appreciated and used for pathophysiological treatments.[5,6] It has been found that this plant possesses many functions, including antimicrobial, antioxidant, analgesic, anti-inflammatory, anti-cancer, as well as wound healing properties.[7,8,9] In addition, *T. polium* is used as a plaster constituent to treat wounds.[10,11] Furthermore, assays showed that *T. polium* inhibits calcium oxalate nucleation and aggregation in a dose-independent manner, supporting its traditional use in preventing or treating kidney stones in Jordan.[12] In Algeria, we observed a decline in the natural reproduction of *T. polium* at the sampling site, likely due to uncontrolled harvesting and harsh climate conditions. Therefore, *in vitro* propagation methods could be a promising way to multiply this plant. This study aims to develop a reliable and effective regeneration protocol that produces plantlets with a bioactive molecule profile and therapeutic effects similar to those of TPMP. Additionally, maintaining a higher yield of this plant is essential to satisfy the population's needs year-round. Consequently, we also conducted a phytochemical screening of polyphenols from the TPMP and TPC of *T. polium* and evaluated the TPRP's toxicity and analgesic properties to support its use in traditional medicine.

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Materials and Methods

Plant material

At the flowering stage of *T. polium*, the plant was gathered in April 2013 at the Ilamen station in Tamanrasset, which is located in the central Sahara of Algeria at longitude 5°29' East and latitude 23°14'.

The plant was recognized based on prior research and received a voucher specimen number (MP-19-4-13) from the herbarium of the Research Laboratory on Arid Zones (LRZA, USTHB), Algiers, Algeria.[13]

Animals

The Center for Research and Development of Saidal (CRD, Algeria) generously supplied Swiss Albino mice with a weight of 20 ± 5 g. The laboratory animals were fed a pellet diet and were given water as needed. The mice were housed and cared for in a controlled environment with a temperature range of 24 ± 1 °C, 12-hour light/dark cycles, and a constant humidity level of 50%. Obtaining approval from the national committee for evaluation and programming of university research ensured that all experimental procedures followed Directive 86/609/EEC.

Explant preparation and culture medium

Callus induction was carried out using apical buds, nodals, leaves, and flowers.[8] A number of growth regulators were added to Murashige and Skoog (MS) medium in order to induce callus. [14] Brown friable calli were the only ones sent to a washing medium devoid of plant growth regulators after 30 days, and they remained there for an additional 20 days. Then, they were transferred to a regeneration medium resembling the callus induction medium prepared without auxins and containing only benzyl adenine (6-Benzylaminopurine, BAP) at 0.1, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/L. The cultures were incubated at 28 ± 2 °C under the influence of a 16/8 h photoperiod using cool white fluorescent tubes (OSRAM L 36W/765). The histological study was performed on serial paraffin sections of the obtained calli to determine their nature and structure.[15,16]

Phenolic compounds extraction

All phytochemical tests were performed on the powder of TPMP, TPC and TPRP. The extraction of phenolic compounds was conducted and comprised a hot hydrochloric acid hydrolysis (HCl, 2 N, 80 mL) of 1 g powder and its exhaustion with diethyl ether, which causes the release of aglycones (flavones-flavonols) and phenolic acids (EthEx).[17,18] The extraction of flavonoid glycosides for TPRP was based on the protocol of Harborne.[19] It allows the extraction of glycosides (O-glycosides and C-glycosides). It consists of maceration of 1 g of TPRP in 100 mL of a hydro-alcoholic solution (70:0, v:v). This maceration lasted for 48 h, followed by dry evaporation of the latter in a rotavapor (Heidolph, Germany). The dry residue was subsequently retrieved in 100 mL of boiling distilled water. After cooling, the mixture was transferred to a separating funnel with 50 mL of n-butanol. The butanolic epiphase formed by the brownish-colored upper fraction containing flavone glycosides (HetEx) was recovered and evaporated. Before being stored in the fridge for future research, the dry residues were suspended in 5 mL of methanol.

High-Performance Liquid Chromatography analysis

A Waters Alliance 2695 chromatography system, equipped with a UV detector and a Diodes Surveyor Bars, was used to perform high-performance liquid chromatography (HPLC). The Discovery HS C18 column (20 x 4.6 mm, 5 µm) was employed for the chromatographic analysis. The samples were analyzed using a gradient: 1-10 minutes in solvent system B (30% B), 10-20 minutes in solvent system A (0.1% phosphoric acid in water), and 20-60 minutes in solvent system B (from 40% to 100% B). Detection was performed at 260 and 365 nm, with a solvent flow rate of 1 mL/min.

Acute toxicity test

The acute oral toxicity of the derived plantlets was assessed in male and female mice, following the OECD guideline-423 with minor changes.[20] The mice were evenly distributed into eight groups (n=10, 5 males and 5 females). The animals had an 18-hour fasting period before the studies and were provided with TPC infusion at a dosage of 2.0 g/kg body weight, although the control group received merely 0.5 mL of saline solution. Food and drink were withheld for an additional 3 to 4 h after administration. The animals were meticulously monitored for 4 h and thereafter examined once daily throughout the course of a fourteen-day monitoring period to determine mortality. Notable behavioral changes observed included tremors, hyperactivity, ataxia, convulsions, lethargy, salivation, diarrhea, sleep difficulties, and coma.[21]

Analgesic activity

The *in vivo* peripheral analgesic effect of the obtained TPRP was studied in mice after inducing pain using chemical stimuli.[22,23] The animals were fasted for 16 h prior to the test. Four batches of 6 mice each were used. The control group received only physiological saline water (0.5 mL/20 g), whereas, for the other batches, we administered EthEx (batch 1) and HetEx (batch 2) to the mice, each with doses of 300 and 500 mg/kg b.wt. The last batch of mice received paracetamol at 300 mg/kg as a reference product. After 30 minutes of the administration of all products, the animals received an intraperitoneal injection of acetic acid (0.6 %, v/v) in physiological saline solution (10 mL/kg b.wt). The number of cramps experienced by each mouse was recorded for fifteen minutes following the injection, five minutes later. The analgesic activity was expressed as the percentage of pain inhibition or the percentage of protection for each group. The means of the groups treated with the extracts and paracetamol were compared with those of the control group, which was treated with physiological saline water. The percentage of protection was calculated according to the following formula:

$$\% \text{ Protection} = \frac{1 - we}{wt} \times 100$$

We: Average number of cramps in mice in the test batch (treated). Wt: Average number of cramps in mice in the control batch (non-treated).

Statistical analysis

The results are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. Differences were considered statistically significant when the *P*-value was below 0.05.

Results and Discussion

Regeneration of in vitro regenerated plantlets

The organogenic type of calli obtained on the explants of *T. polium* was the subject of the leaf shoot regeneration on their surface and then the conversion of the latter into plantlets (Figure 1). The observation of the histological sections of the obtained calli revealed that the calli initiated on the apical buds are organogenic. We observed a parenchymatous zone without clear differentiation, in which we can see, in places, a beginning of individualization of the peripheral meristematic cells, showing the organization of numerous domes or meristematic ridges (Figure 2 a). The first histological event observed immediately after the transfer of these organogenic calli into the regeneration medium is the histological differentiation of the meristematic cells located at the level of the domes (Figure 2 b), suggesting a process of budding and leading to the formation of adventitious shoots at the peripheral calli. The sequential appearance of buds accompanied by shoots was then observed (Figure 2 c and d). Explants of apical buds had formed the majority of calli with morphogenesis, which allowed us to isolate a budding strain. Callus, from other explants, did not have caulogenic capacities. The stimulation of the appearance of small foliar parts was possible on the surface of the obtained organogenic calli. The highest rate of regeneration of 100% was recorded in a BAP-enriched medium of apical bud explants, particularly at the doses of 1.5 and 2.0 mg/L. [9] This regeneration rate at optimized BAP doses further highlights the efficiency of the protocol and the responsiveness of apical bud explants, confirming juvenility and explant type as determinant factors in tissue culture success.[8,]

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In vitro regenerated plantlets from callus

Three-month-old organogenic calli were selected and subcultured in a BAP-rich medium; after one month of culture, we observed young shoots bearing leaves appearing as tufts on the entire surface of calli reaching 2 cm in size in both width and length.

Figure 1: Different callus morphotypes from *T. polium*. a. Compact yellow callus, b. Nodular green callus, c. Friable green callus. Scale bar = 0.5mm.

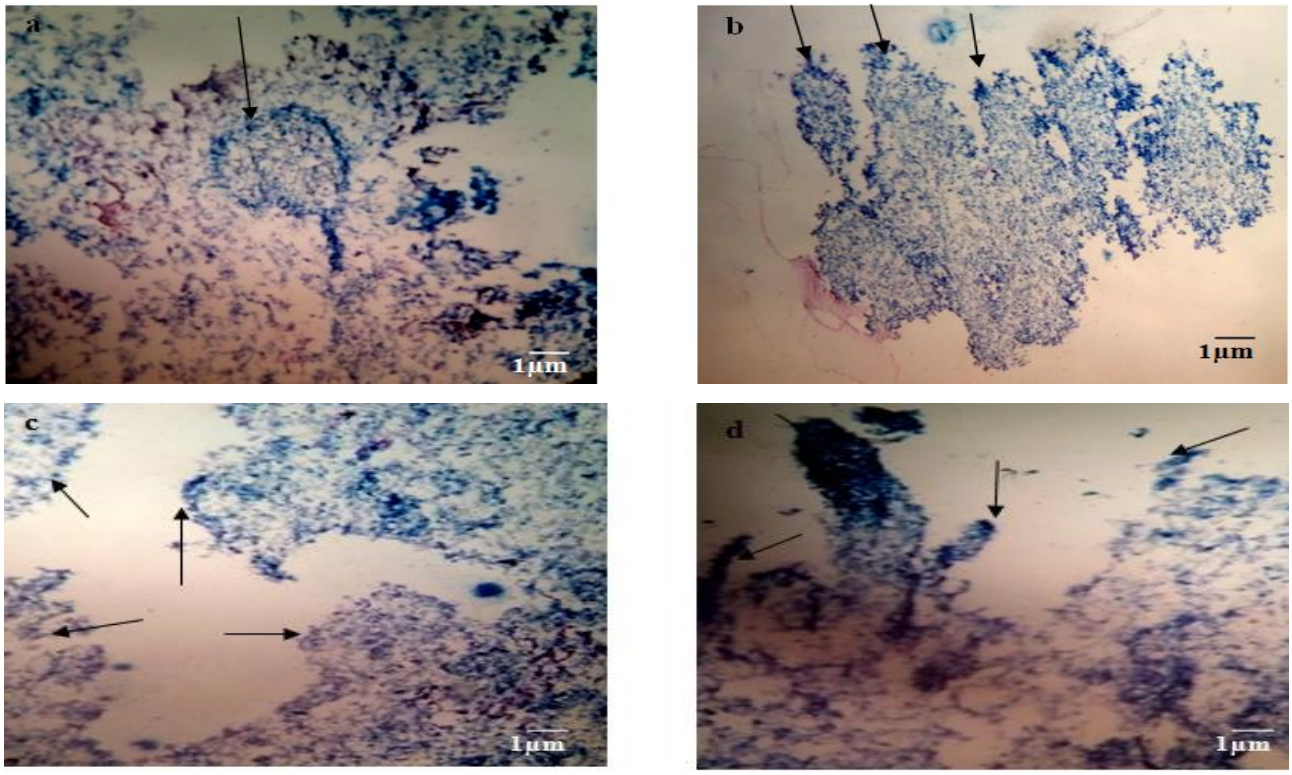

Figure 2: Histological aspects illustrating the different stages of regeneration from organogenic calli in *T. polium*. a. Differentiation of meristematic cells at periphery, b. Formation of adventitious shoots at periphery, c. Buds development with leaf primordia, d. Explants of apical buds.

Shoots are erect, thin, transparent, branched, bear stalked leaves, and are bright green (Figure 3 a, b and c). These micro-shoots are then separated from calli and individually subcultured on a fresh culture medium of similar composition to the initial regeneration medium to stimulate plantlet development. After successful multiplication for one and a half months of culturing in the regeneration medium, the young plantlets were subcultured and transferred to different rooting media rich in NAA for one month for the induction of roots (Figure 3 d, e and f). Table 1 shows that culture media supplemented with 1.5 and 2 mg/L BAP were clearly the most effective—though not significantly different from each other ($P > 0.05$)—in inducing young shoot formation over the entire surface of calli derived from apical buds. Both media achieved a regeneration rate of 100%. The medium containing 1.0 mg/L BAP differed significantly from the others and favored regeneration through the neoformation of adventitious buds, with a mean value of 90.40 ± 1.80%. In contrast, the media enriched with 0.5 and 2.5 mg/L BAP were also significantly different from the remaining treatments, producing lower budding percentages of 80.10 ± 4.90 and 83.30 ± 3.80, respectively. These percentages remain significantly important compared to those recorded in the callus grown in the medium supplemented with 0.1 mg/L, which was not very effective because we obtained the lowest percentage of regeneration in the callus resulting from the apical buds, which was 71.80 ± 4.90. We also noted the superiority of the medium composed of the lowest used concentration of BAP, which is 0.1 mg/L, in the initiation and development of the adventitious buds in the callus derived from nodal fragments; the

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obtained rate was 50.10 ± 2.50, followed by a rate of 50.02 ± 2.60 obtained in the culture medium at the concentration of 1.5 mg/L of BAP. We noted that the regeneration potential in calli obtained from nodal explants cultured in nutrient media containing BAP at concentrations of 2.0 and 2.5 mg/L was in favor of bud neoformation on their surfaces without significant differences.

Table 1: Regeneration rate of *in vitro* plantlets

BAP	0.1 mg/L	0.5 mg/L	1 mg/L	1.5 mg/L	2 mg/L	2.5 mg/L
Organ						
Callus from apical buds	71.80±4.90^{D}	80.10±4.90^{C}	90.40±1.80^{B}	100.00±0^{A}	100.00±00^{A}	83.30±3.80^{C}
Callus from nodals	50.10±2.50^{H}	16.40±0.90^{H}	34.60±2.20^{G}	50.02±2.60^{E}	44.70±2.50^{E,F}	40.30±0.90^{F,G}
Callus from flowers	0±00^{I}	0±00^{I}	0±00^{I}	0±00^{I}	0±00^{I}	0±00^{I}
Callus from leaves	0±00^{I}	0±00^{I}	0±00^{I}	0±00^{I}	0±00^{I}	0±00^{I}

Values are expressed as mean ± SD, (n = 6), each measurement is repeated 3 times, values with different letters are statistically different ($P < 0.05$).

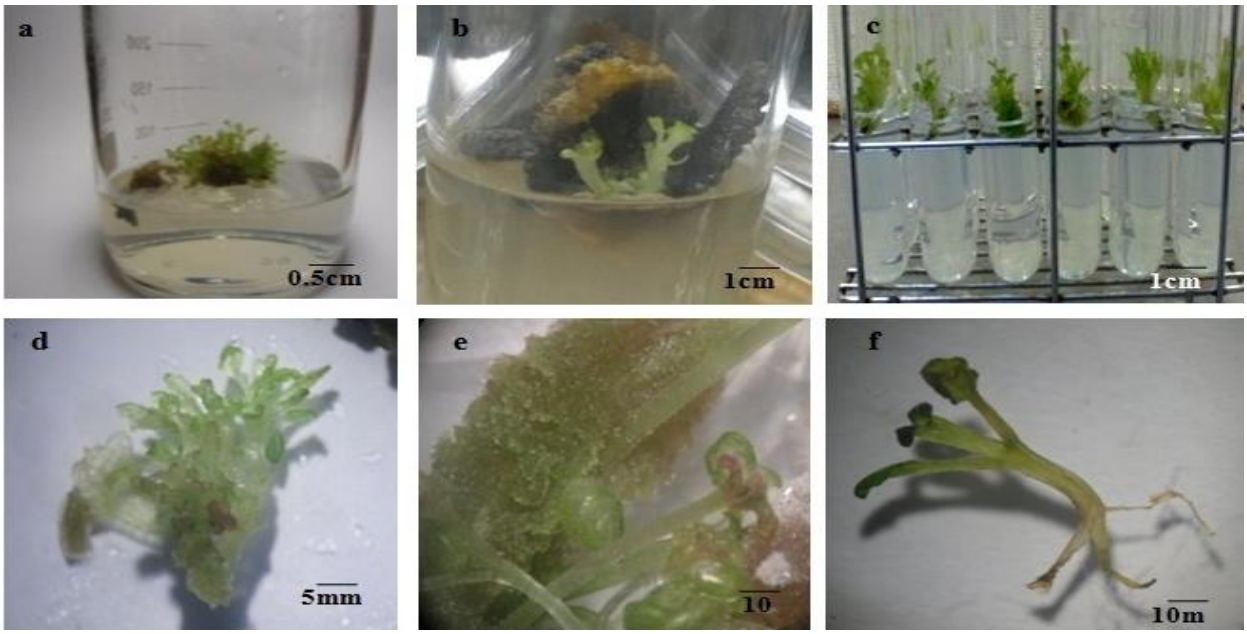

Figure 3: Developmental stages of *T. polium* regenerated plantlets. a, b and c. Shoot regeneration, d. Tufts, e. Plantlets hardening inside the culture room, f. Root induction.

Thus, these media showed an average budding rate of 44.70 ± 2.50 and 40.30 ± 0.90, respectively. The results also show that BAP was not effective in callus formation from flower fragments and leaves. The efficiency of *in vitro* regeneration of a plant can be influenced by several factors, such as genotype, explant type, and various combinations of growth regulators. In general, direct shoot regeneration of plantlets is achieved when employing apical buds as explants and is positively impacted by BAP and IAA.[24]

HPLC analysis

The HPLC analysis of the different extracts is shown in Figure 4 and Table 2. The comparison between the retention times of the extract samples and the injected standards under the same conditions. This analysis allowed the identification of one peak that appeared at the wavelength of 260 nm, corresponding to gallic acid (24674.43 mAU), which belongs to the hydroxybenzoic acid class in the TPRP. On the other hand, at the wavelength of 360 nm, three flavonols named quercetin, isorhamnetin and kaempferol were identified with a total absolute content of 4770.63 mAU. For flavones, we noted the presence of luteolin, tricin and apigenin, with a total absolute content of 34492.35 mAU. The HPLC profile at 365 nm showed one peak of an analogous compound to cinnamic acid, which is n-hydroxycinnamic acid, with an absolute content of 1509.68 mAU. For TPC, we have also identified gallic acid (9480.00 mAU) at 260 nm. The class of flavonols is represented by quercetin (1758.90 mAU), and the class of flavones is represented by apigenin (4329.00 mAU). These results resemble those found in the TPMP. Flavones also dominated TPC. For phenolic acids, a peak corresponding to cinnamic acid (5139.12 mAU) was identified.

For the TPRP, the analysis revealed the presence of two hydroxybenzoic acids: gallic acid (16.77 mAU) and protocatechuic acid (1.02 mAU). In addition, hydroxycinnamic acid (63.61 mAU) has been detected. As for flavonols, we identified quercetin, kaempferol and isorhamnetin, which have a total absolute content of 262.41 mAU. We also identified apigenin, luteolin and tricin as flavones with a total absolute content of 120.04 mAU.

This study identified phenolic acids and aglycones (flavones and flavonols) as key bioactive phytochemicals present in adult plant powder, calli, and *in vitro* regenerated plantlets. The results reveal substantial variability in the contents of phenolic compounds, hydroxybenzoic acids, cinnamic acids, flavones and flavonols across the three biological models. Quantitatively, the adult plant powder contains the highest proportions of phenolic acids, particularly hydroxycinnamic acid and gallic acid. In contrast, several components were absent or present in markedly lower amounts in the calli and regenerated plantlets compared to the adult plant. This can be explained by the secondary metabolism destabilization during the *in vitro* culture because of the non-organized histological tissues that are composed of meristematic cells and as a consequence of the non-fixed genomic

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expression.[25] Gallic acid was detected in the HPLC profiles of the adult plant, callus, and in vitro regenerated plantlets, whereas protocatechuic acid was identified exclusively in the *in vitro* regenerated plantlets.

Table 2: HPLC data of the extracts of *T. polium* L. subsp. *geyrii* Maire powder detected at 260 and 365 nm.

	Peak	Retention time (min)	Identified compounds	Absolute area (mAU)
Mother plant	1	6.079	Gallic acid	24674.43
	2	33.143	Quercetin	1686.40
	3	34.384	Luteolin	2540.00
	4	35.235	Kaempferol	1073.03
	5	36.461	Isorhamnetin	2011.20
	6	38.541	Tricin	755.35
	7	39.644	Apigenin	31197.00
	8	41.459	Hydroxycinnamic acid	1509.68
4-month-old callus	1	6.080	Gallic acid	9480.00
	2	31.778	Quercetin	1758.90
	3	38.587	Apigenin	4329.00
	4	40.888	Hydroxycinnamic acid	5139.12
In vitro regenerated plantlets	1	6.090	Gallic acid	16.77
	2	10.076	Protocatechuic acid	81.02
	3	18.450	Quercetin	21.39
	4	20.037	Luteolin	25.28
	5	22.320	Kaempferol	105.02
	6	31.710	Isorhamnetin	136.00
	7	35.850	Tricin	49.65
	8	37.127	Apigenin	45.11
	9	38.709	Hydroxycinnamic acid	63.61

Moreover, we observed that callus tissues produced gallic acid in large amounts compared to *in vitro* regenerated plantlets, and on the other hand, the differentiation of callus into *in vitro* regenerated plantlets caused the predominance of protocatechuic acid at the expense of gallic acid These two components have the same biosynthesis pathway, and a hypothesis of an alteration of the enzymatic regulation of their production was given by different authors.[27,28] For young plantlets of two months *in vitro* culture, cells produce at first protocatechuic acid, which plays the role of a precursor of gallic acid with the reorientation of their biosynthesis pathway in adult plants. In fact, the absence of some components in the callus is most likely influenced by factors such as age, culture medium, and environmental conditions. A qualitative analysis of several species from the Lamiaceae family led to the identification of compounds including luteolin, glucoside, and apigenin.[29] Several factors, including the stage of development, the soil, and the ambient conditions, determine the kind and concentration of these chemicals.[30]

Acute toxicity assessment

The toxicity test findings indicated that the oral administration of TPRP at doses up to 2.0 g/kg body weight caused no signs of toxicity or behavioural changes in mice during the first 4 hours following administration. Throughout the 14-day trial, no death was detected in either sex of mice; hence, the LD50 of TPRP exceeds 2.0 g/kg of body weight.

Also, protocatechuic acid was reported in different plant extracts, including *Satureja graeca* L. ethyl acetate extract.[26]

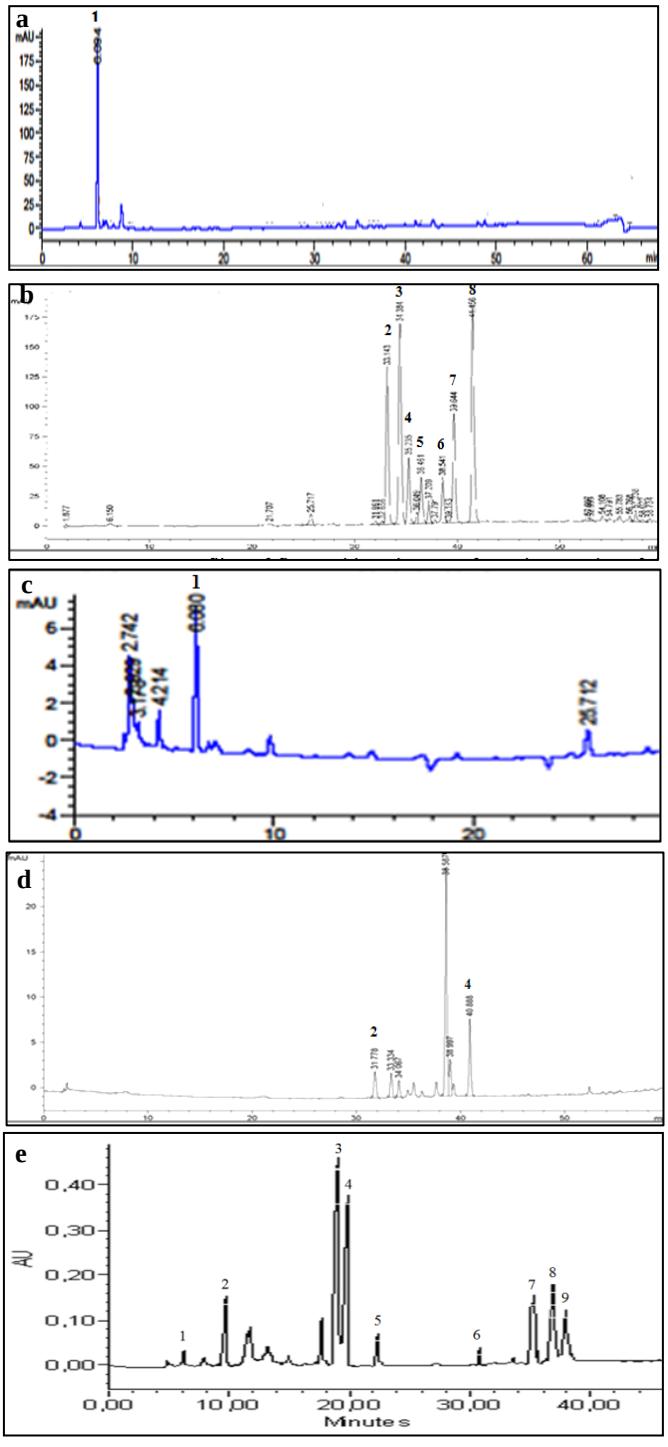

Figure 4: HPLC profiles of flavonoid aglycones from the powder of the adult plant of *T. polium* L. subsp. *geyrii* Maire detected at 260 (a) and at 365 nm (b), 4-month-old callus detected at 260 (c) and 365 nm (d) and *in vitro* regenerated plantlet at 365 nm

The toxicity of *in vitro* regenerated plantlets of *T. polium* L. subsp. *geyrii* Maire has never been evaluated before; therefore, this is the first report. The study showed that the *in vitro* regenerated plantlets are non-toxic and can be used in remedy preparation, which ascertains their use by the local Tuareg population of Tamanrasset.

Analgesic activity of in vitro regenerated plantlets

The results of the analgesic effects of the EthEx and HetEx extracts of the TPRP are summarized in Table 3. The control group that received physiological saline water had a writhing average of 57 ± 1.8 over 15 min. The recorded values indicated that the mice in this group did not exhibit any pain inhibition during the test. The mice of the control group, therefore, had the highest number of cramps. Oral administration of paracetamol at a dose of 300 mg/kg significantly reduced acetic acid-induced contortions ($P < 0.001$) compared to the control group (31 ± 1.8), achieving a 50% protection rate. Concerning the extracts, we noticed that the analgesic activity was dose-dependent. Knowing that the EthEx extract containing the flavonoid aglycones and the phenolic acids proved to be more effective than the HetEx, we recorded the best rate of protection with the dose of 500 mg/kg (90 %) and a highly significant decrease in cramping ($P < 0.001$), compared with the control and reference groups.

Table 3: Analgesic effect of the *in vitro* regenerated plantlet extracts of *T. polium* L. subsp. *geyrii* Maire on abdominal contractions in mice

Groups	Doses (mg/kg)	Writing number	Protection percentage (%)
Control	/	57±1.8$^{£}$	-
Paracetamol	300	29±1.4$^{***£}$	50
EthEx	300	8.16±4.8$^{***£}$	86
	500	5.88±3.2$^{***£}$	90
HetEx	300	15±0.8$^{***£}$	74
	500	11.33±2.1$^{***£}$	81

The values are expressed as mean ± SD, ***$P < 0.001$: significant compared to control, £ $P < 0.001$ compared to reference (one-way ANOVA followed by Tukey's test), EthEx: diethyl ether extracts, HetEx: hydro-alcoholic extract.

The obtained results of the analgesic potency of the *in vitro* regenerated plantlets showed that diethyl ether (EthEx) and hydro-alcoholic (HetEx) extracts had a significant analgesic effect by reducing the number of abdominal contortions at all tested doses. This suggests that they have compounds that act on the same mechanism as paracetamol, thereby inhibiting COX-1 and COX-2, preventing prostaglandin synthesis. [31] Previous studies have evaluated the biological activity of callus aqueous extract and various adult plant extracts of *T. polium* (diethyl ether containing flavonoid aglycones, hydro-alcoholic with C- and O-glycosides, and butanolic with C-glycosides).[8,9] Results demonstrated that callus infusion at 500 mg/kg body weight outperformed adult plant extracts, with the diethyl ether extract from adult plant powder also showing notable effectiveness.[8] The HPLC results of the diethyl ether extract of the *in vitro* regenerated plantlets revealed the presence of quercetin, a molecule reported by several authors to have analgesic activity.[32,33,34] In addition, quercetin was reported to possess strong binding affinity and stability with COX-2, highlighting its promise as an anti-inflammatory agent.[35] The significant analgesic effect observed could be the result of the synergistic action of the various phenolic compounds that the diethyl ether extract of the *in vitro* regenerated plantlets contains, mainly isorhamnetin and kaempferol, known for their peripheral analgesic effects.[36]

Conclusion

The comparison between TPC and TPRP extracts with respect to their phenolic compound profiles revealed that TPRP extracts contained the lowest quantities. It should be emphasized that the current process is far from optimized. Adjusting certain culture conditions, such as light intensity and/or duration, pH variation, or the use of elicitors to enhance biomass production, may prove beneficial. These considerations open promising avenues for more in-depth investigations aimed at isolating and identifying bioactive molecules.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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