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Enhancing bioactive potential by growth regulators in callus of *Mentha longifolia* L. leaves for anti-inflammatory and analgesic activities

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Mentha longifolia L., popularly known as Mint, is a medicinal herb against inflammation and analgesic pains caused by venom of snakes and scorpions. Its leaves are commonly used by nomads of Ahaggar to treat external wounds. Unfortunately, this medicinal plant is less accessible in its natural zone. Hence, here, we propose a biotechnological approach to induce callus yielding the same effects. Callus was induced from young leaves cultivated on modified Murashige and Skoog solid medium supplemented with 30 g L^{-1} sucrose, 100 mg glutamine and growth regulators under dark conditions. Aqueous and C-glycoside extracts of plant and callus, respectively were prepared and analyzed by HPLC-DAD/LC-ESI-MS. The study of acute toxicity at 2000 mg kg^{-1} followed by the anti-inflammatory activity assessment using carrageenan-induced paw edema and the analgesic activity of acetic acid induced writhing were performed on mice. Compared to the control, the trials showed 79% higher rate of friable callus induced on 2,4-D and BAP. No mortality or signs of toxicity were observed in mice. Unexpected higher anti-inflammatory and analgesic activities by callus extracts can be attributed to their phenolic composition.

Keywords: Ahaggar nomads, Anti-inflammatory, Antianalgesic, Callus, Herbal, Inflammation, Mint leaves, Venom

Mentha longifolia L., is a perennial plant commonly called mint belonging to Lamiaceae (ex: Labiateae), found in shadow around water areas of Gueltates Afilal under the mountains of Ahaggar (southern Algeria) due to its much branched stolons (1-2 m height), long stems surrounded by wide grayish green oblong elliptical leaves and hermaphrodite purple flowers[1]. This aromatic herb, is mainly used by traditional healers against inflammation (cold, sore throat, fever), gastrointestinal diseases (Antispasmodic, antidiarrhoeal) and analgesic troubles (wounds, snakes bites and scorpion stings)[2-5]. In arid zones, where aboriginals suffer from several ailments (drought, warmness and abundance of reptiles) without any accessibility to medical drugs expensive[6,7]. *M. longifolia* L. extracts also exhibit significant antioxidant, hepatoprotective and anticancer activities[5,8,9]. *Mentha* spp. have rich polyphenols content which are responsible for their medicinal properties[10,11] and hence are used traditionally for centuries as herbal tea, fresh vegetable and condiment[12,13].

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The inflammatory pain is often treated with non-steroid anti-inflammatory drugs (NSAIDs), such as aspirin, diclofenac or ketorolac which exert the analgesic effect through a variety of peripheral and central mechanisms[14]. However, several chemical drugs revealed their common relationship to increased risk of gastrointestinal bleeding in both acute and chronic therapy[15]. Hence, there is a need to develop safe new anti-inflammatory and analgesic natural product without any side effect. Indeed, earlier studies have highlighted that leaves are used to treat inflammatory disorders and reduce abdominal pains by infusion and decoction forms[16,17].

In the last decades, plant tissue culture works have contributed to development of considerable potential bioactive secondary metabolites that serve as natural antioxidant and active substances for human health [18-20]. The increased demand for this herb, has encouraged researchers to develop new *in vitro* culture methods as biotechnological approach to induce callus from leaves, then multiplied for increased production of active principles, and there by save this wonder herb in its natural wild habitat.

In this context, we too have explored obtaining callus from mint leaf explants for possible alternate

source of natural mint from the wild. We have also evaluated the bioactive composition by HPLC-DAD/ LC-ESI-MS analysis, and also the anti-inflammatory and analgesic activities of aqueous and C-glycoside extracts of the plant and callus on experimental animal.

Materials and Methods

Chemicals, such as 2,4-Dichlorophenoxyacetic acid (2,4D), 6-benzyl aminopurine (BAP), naphthalene acetic acid (NAA), Isopentenyl adenine (IPA), α-D-glucopyranosyl-(1↔2)-β-D-fructofuranoside (sucrose), 2,5-diamino-5-oxopentanoïque (glutamine), agar were purchased from Sigma Chemicals ALDRICH (USA); CH_3COOH (acetic acid), butan-1-ol (n-1 butanol), hydrochloric acid (HCl), mercuric chloride ($HgCl_2$) from Biochim SARL (Algeria); and N-(4-hydroxy-phenyl) acetamide (paracetamol), $C_{24}H_{36}O_{25}S_2^{-2}$ (carrageenan) (acetyl-salicylic acid) $C_9H_8O_4$ (aspirin) were obtained through Research and Development CRD-SAIDAL collaboration.

Sample collection

Adult plants of *Mentha longifolia* L. were freshly harvested in March (2013-2015) from "Gueltates Afilal", recognized as one of the 42 wet areas by Ramsar Convention facing desertification in the arid areas of Ahaggar[21], distributed on 20900 ha at 2018 m altitude, 23◦08'43.1s North latitude 005◦43'48.8s East longitude in Tamanrasset, influenced by the Mediterranean climate with 15-20°C (from November to February) and a tropical climate with 40-42°C (from May to September).

The collected plant specimen was authenticated at the National Institute of Forestry Research of Tamanrasset (INRF), with the help of Flora of Sahara[22]. The voucher specimen was deposited in the Herbarium of Maire (N°34. LRZA, Algiers).

Preparation of extracts

Two different leaf extracts were prepared to carry out the chromatographic analysis and biological activities. The aqueous extracts of the aerial parts and callus were separately obtained using infusion method. An amount of 0.4 g of each (crushed shade dried plant; grinded fresh callus in a mortar) was extracted using 10 mL of boiling water for 20 min, and then filtered. The C-glycoside extracts were recovered, by acid hydrolysis. One g powder, 80 mL HCl 2N) at 40°C for 40 min, and then filtered, 25 mL of butanol (1N) was added and then the C-glycosides fractions collected, air dried at 27°C. The residual was dissolved in physiological water 0.9%[23].

Callus induction

Whole leaf branches (5 cm) of *M. longifolia* L. freshly collected were taken, washed by soaking in aqueous "Isis detergent" added with 2 drops of sodium hypochlorite (NaOCl) at 12°C for 2 min, gently rinsed by tap running water for 20 min. The leaves were isolated, transferred to Alcohol 70% for 1 min, and then placed in mercuric chloride $HgCl_2$ at 0.5‰ for 4 min, then rinsed thrice for 10 min each by sterilized distilled water[24]. Leaf explants of 0.5 to 1 cm were cultured in "supine position" in Petri dishes 9 mm diameter containing 20 mL of (MS) medium[25], added of 30 g of sucrose, 7 g of agar, 100 mg of glutamine and various combinations of growth regulators as follows: M1 (2, 4-D 2. 2. 10^{-6}; BAP $4.5.10^{-6}$ M), M2 (2, 4-D $2.2.10^{-6}$; IPA $14.8.10^{-6}$ M), M3 (NAA $5.4.10^{-6}$; BAP $4.5.10^{-6}$ M). The pH of the medium was adjusted to 5.7 before autoclaving at 120°C for 20 min. The cultures were incubated at total darkness at 25°C. Daily observations were made, and then contaminated explants were removed. Explants with primary callus were subcultured on the same media composition until high multiplication to express the rate (%).

Animals

Young albino mice of both male and female sexes (20-30 g) from Toxicology laboratory of CRD-Saïdal (Algiers, Algeria), were maintained under standard environmental conditions of animal house (temperature, 24±2°C, and 12 h light/dark cycle) with a free access to croquettes scheme and tap water *ad libitum*. The experiments were performed following OECD Guideline-423 (Organization for Economic Co-operation and Development). This procedure is reproducible, uses very few animals and is able to rank substances to acute toxicity testing methods.

Qualitative and quantitative phytochemical analysis

The phytochemical analysis of phenolic compounds was determined by the application of HPLC-DAD and LC-ESI-MS techniques for identifying exact molecular weight of compounds from a small amount of plant sample extracts with low concentrations. Chromatogram profiles were determined in all the samples.

Each compound was identified by its retention time and by compared the mass spectrum of the component in aqueous plant extract with the mass spectrum of reference standard at 280 and 365 nm

using a flow rate of 1.5 mL/min on a column = Hypersil BDS-C18, 5 µm, 250 × 4.6 mm at 30°C. The gradient consists of two phases, Phase A (0.2% acetic acid) and Phase B composed of acetonitrile (HPLC). LC-ESI-MS analysis was performed using a LCMS-2020 quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an electro spray ionization source (ESI) and operated in negative mode (Arid Research Institute (IRA) Medenine, Tunisia).

Acute oral toxicity

The acute oral toxicity was determined[26], 3 groups of 3 female mice each (20-23 g) were fasted 12 h overnight. At the day of the experiment, each group of mice received a dose of 2000 mg Kg^{-1} body wt. by oral gavage of solution as follows: experimental Group I (plant aqueous extracts), experimental Group II (callus aqueous extracts) and control group (physiological water 0.9%).

All abnormal behavioural changes were recorded before and after dosing at least twice a day for 14 days. Signs and/or symptoms of toxicity including mortality were noted, to estimate the LD_{50} (medium lethal dose)[27] for each dose did not cause any mortality.

Pharmacological studies

Two pharmacological (Anti-inflammatory and analgesic) activities were achieved on Swiss albino mice to check the efficiency of plant and callus extracts. The acute oral toxicity test was performed at a single dose to ascertain the plant material tested in further experiments.

Evaluation of anti-inflammatory effect

Carrageenan induced paw edema was analyzed in mice model[28], 6 groups of 6 albino mice each were prepared as follows: Group I control (physiological water 0.9%), Group II standard drug (10 mg kg^{-1}, aspirin) treated, Group III (1 g kg^{-1}, plant aqueous extracts), Group IV (250 mg kg^{-1}, plant C-glycoside extracts), Group V (1 g kg^{-1}, callus aqueous extracts) and Group VI (250 mg kg^{-1}, callus C-glycoside extracts). About 0.5 mL of different extracts was administered orally, 30 min after, and then 0.025 mL of 1% carrageenan was injected in the left hind paw to causing an edema 3 h later, the inflammatory responses were developed and measured. All animals were sacrificed; the right hind paw was served as control to comparing with hind paw treated. The anti-inflammatory activity was expressed as percentage calculated according to the following formulas:

$$\text{Percentage of increase in paw volume} = \frac{V1 - V0}{V1} \times 100$$

V1: the weight of paw treated; V0: the weight of the right hind paw untreated.

$$\text{Percentage of reduction of paw edema} = \frac{PIc - PItr}{PIc}$$

PI_c: Percent reduction of paw edema of control; PI_{Tr}: Percent reduction of paw edema treaded.

Evaluation of analgesic effect

The acetic acid-induced writhing test was analyzed in mice model[29]. Albino mice were divided into 6 lots of 6 mice each (control, reference and assays groups), Group I control (physiological water 0.9%), Group II standard drug (100 mg kg^{-1}, paracetamol) treated, Group III (1 g kg^{-1}, plant aqueous extracts), Group IV (250 mg kg^{-1}, plant C-glycosides extracts), Group V (1 g kg^{-1}, callus aqueous extracts) and Group VI (250 mg kg^{-1}, callus C-glycosides extracts). At the day of the experiment, all mice received separately a volume of 0.5 mL of extracts tested. 30 min later, about 0.6% acetic acid solution (0.2 mL) was injected intraperitoneally and the total number of writhes was noted after 5 min. All animals were monitored for 30 min. The writhing response consists of the contraction of abdominal muscle followed by hind limb extension. The percentage of writhing inhibition was calculated with the following equation:

$$\% \text{ Inhibition} = \frac{\text{Mean N. of writhes in CG} - \text{Mean N. of writhes in TG}}{\text{Mean N. of writhes in CG}} \text{ X } 100$$

N: number. CG: control group. TG: treated group.

Statistical analysis

Results obtained were reported as means± SD. The mean values were compared using t-test. The experiments were performed in triplicates, differences between means were determined by one way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons with control. A value of $P < 0.05$ was considered statistically significant.

Results & Discussion

Obtention of callus

A protocol for callus induction and expression of anti-inflammatory and analgesic via leaf segment-derived callus of *Mentha longifolia* were developed.

Under *in vitro* culture conditions, leaf explants showed several callus masses varied with media and time of culture. Depending of growth regulators, the highest rate callus (79%) was observed on M1 with $2{,}2.10^{-6}$ M 2,4-D and $4.5.10^{-6}$ M BAP followed by that of 76% on M3 with $5.4.10^{-6}$ M NAA and $4.5.10^{-6}$

M BAP and 41% on M2 with $2.2.10^{-6}$ M 2, 4-D and $14.8.10^{-6}$M IPA (Fig. 1A).

The results expose that the association of auxins-cytokinins is required for callus induction; their combination may play a decisive role in the estimation of the rate of callus obtained. Indeed 2,4-D/BAP appears more effective to the callus induction than 2,4-D/IPA and NAA/BAP. Concerning the nature of callus formed, most of them were friable with variable colours; creamy, yellowish to brownish with necrosis on whole explants with time (13 weeks) (Fig. 1 B and C). Earlier reports showed, creamy, fragile and poor rate of callus initiated by cell suspensions of *Mentha longifolia* on 0.5 mg L^{-1} 2, 4-D and 1 mg L^{-1} BAP[2]. On the other hand, creamy and friable callus with small necrosis onto vegetative explants surfaces of this plant was revealed[30]. The colour of callus is mainly influenced by location of phenolic secondary metabolites in cells. The yellowish callus expresses that phenolic compounds are in vacuoles, whereas, the accumulation of phenolic compounds in the cytoplasm makes callus brown[20].

Phytochemical composition

HPLC method validation

Table 1, presents a range of method validation parameters. Results showed the correlation coefficients larger than 0.996 showing a good relationship between the peak areas and concentrations. LOD (Detection limits) of phenolic compounds in the sample tested were found to be satisfactory. The method precision was validated by the analysis of a spiked sample. The recoveries of these standards were between 98.16 and 100.12%.

Determination of Phenolic compounds

Comparative HPLC-DAD and LC-SEI-MS analyses (Fig. 2 A and B) of aqueous and C-glycosides extracts of *M. longifolia* have shown accordance between the amounts of phenolic compounds in plants and callus. A comparative analysis of Table 2, shows that both of aqueous and C-glycosides extracts of *M. longifolia* contain all phenolic compounds revealed with different amounts. Nevertheless, the content in aqueous extracts generally is more than in C-glycosides extract. This is in agreement with earlier studies which noted the presence of high phenolic content in aqueous extracts of *M. longifolia* and other *Mentha* species[31].

Although, the content of phenolic compounds in callus aqueous extracts is higher than in adult plant, the amount of t-ferulic acid is lower in callus than in adult plant. Also, callus C-glycosides extracts may contain a high percentage of phenolic compounds

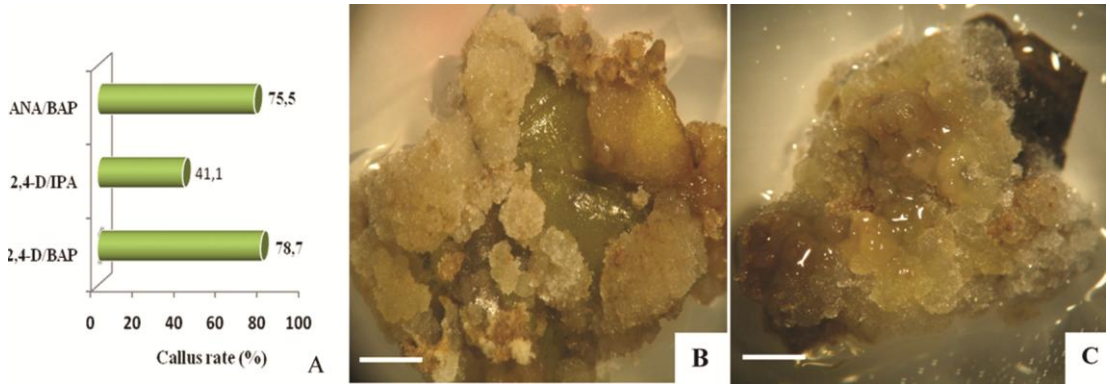

Fig. 1 — (A) Callus rate variation on different growth regulators combinations; and(B & C) *In vitro* callus induction of *Mentha longifolia* L. [Callus grows from leaf explants on, 2, 2. 10^{-6} M2, 4-D and 4, 5.10^{-6} M BAP, at 8 (bar=10 mm) and 13 weeks (bar=16.66 mm)]

Table 1 — Validation parameters of applied method

Compounds	Recovery (%)	Intercept	Slope	Correlation coefficient (%)	RSD (%)	LOD (mg L^{-1})	LOQ (mg L^{-1})
Gallic acid	99.99	-12764.7	639322	0.998	14.704	0.249	0.754
Caffeic acid	98.16	-42044.8	1.62E+06	0.996	22.33	0.384	1.164
Rosmarinic acid	100.12	-10972.9	1.30166 E+06	0.999	7.848	0.076	0.232
t-Ferulic acid	100.35	1362.62	1.01E+06	0.998	10.99	0.249	0.756
p-Coumaric acid	99.99	-11324.6	929069	0.997	26.455	0.172	0.523
Naringenin	100.01	-8690.71	8.85E+06	0.999	8.05	0.433	1.313

[LOD: Limit of Detection, LOQ: Limit of Quantification, RSD: Relative Standard Deviation]

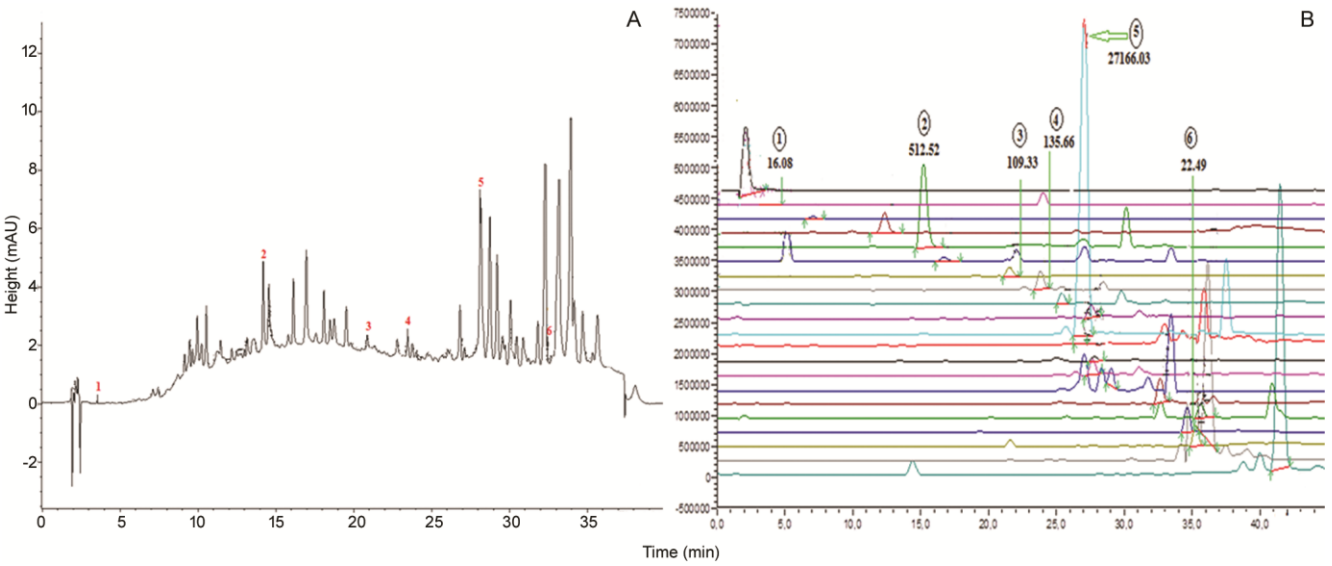

Fig. 2 — Chromatograms of phenolic compounds identified in *Mentha longifolia* L. aqueous extracts. A. HPLC-DAD chromatogram, B. LC-ESI-MS chromatogram. Gallic acid (1), caffeic acid (2), p-coumaric acid (3), t-ferulic acid (4), rosmarinic acid (5), and naringenin (6)

(t-ferulic acid, caffeic acid, p-coumaric acid and naringenin) even if the gallic acid was not detected.

Table 2 — Quantitative analysis for determination of phenolic components in the aqueous extracts and C-glycosides extracts of *M. longifolia*

Compound (Standard retention time)	Aqueous extracts		C-glycoside extracts	
	Plant	Callus	Plant	Callus
Naringenin (34.650)	22.49	73.03	0.286	112.23
p-Coumaric acid (21.560)	109.33	284.98	8.90	66.64
Gallic acid (3.933)	16.08	57.83	17.03	ND
Rosmarinic acid (27.121)	27166.03	34267.36	720.97	118.22
Caffeic acid (15.198)	512.52	633.53	39.63	73.14
t-Ferulic acid (23.801)	135.66	102.09	10.77	228.51

[mg kg^{-1} extract dry weight. ND. Not detected]

In both extracts tested, the highest amount of phenolic compounds was that of rosmarinic acid followed by caffeic acid, t-ferulic acid, p-coumaric acid and gallic acid with naringenin (flavanone), considered as important part of phytochemicals in *M. longifolia*. Indeed, in the aqueous extract, the content of phenolic compounds is higher in callus than in plant, the most abundant phenolic acids were rosmarinic acid (callus: 34267.36 mg kg^{-1}; plant: 27166.03 mg kg^{-1}) followed by caffeic acid (callus: 633.53 mg kg^{-1}; plant: 512.52 mg kg^{-1}), p-coumaric acid (callus: 284.98 mg kg^{-1}; plant: 109.33 mg kg^{-1}) and t-ferulic acid (callus: 102.09 mg kg^{-1}, plant: 135.66 mg kg^{-1}). Note that gallic acid and naringenin were lower as well in callus as in plant extract.

These compounds represent a potential source of natural substances in plants, which have a vital effect in the treatment of population health problems such as, inflammation and fever. In addition, the high content of phenolic compounds in callus of this medicinal plant indicates that this plant material has strong potential health benefits.

According to the results of earlier works, rosmarinic acid is the dominant compound of the phenolic acids of *M. longifolia*[32,30]. It has several biological properties including antiviral, antibacterial, anti-inflammatory and antioxidant activities, its occurrence in plant extract is highly desired[33]. Ozer [8] has shown that infusion of *M. longifolia* yields high content of rosmarinic acid (620.9 mg kg^{-1}), followed by t-ferulic acid (162.7 mg kg^{-1}), caffeic acid (113.1 mg kg^{-1}) and p-coumaric (14.6 mg kg^{-1}). Bahadori *et al.*[34] revealed phenolic compounds in infusion of *M. longifolia* where rosmarinic acid (6260 µg g^{-1}) is the most abundant phenolic component followed by caffeic acid (119 µg g^{-1}), gallic acid (72 µg g^{-1}) and p-coumaric acid (15 µg g^{-1}) but ferulic acid has not identified under their experimental conditions. These investigations show that the amount of rosmarinic acid in both plant and callus aqueous extracts of *M. longifolia* (Algeria) is higher than that of infusion of *M. longifolia* of Iran (6260 µg g^{-1})[34] and of Turkey (620.9 mg kg^{-1})[8]. In contrast, the content of rosmarinic acid in C-glycoside extracts of both plant and callus are lower than that of callus in MeOH 70% extract (21730 mg kg^{-1}) of *M. longifolia* of Poland[30].

In C-glycoside extracts, the content of caffeic and p-coumaric acids are respectively in both plant

(39.633 mg kg^{-1}, 8.909 mg kg^{-1}) and callus (73.144 mg kg^{-1}, 66.647 mg kg^{-1}) was variable than that found in ethanol extract of *M. longifolia* of Iran (caffeic acid: 86 µg g^{-1} and p-coumaric acid: 5 µg g^{-1})[34].

The C-glycosides exhibited a high content of t-ferulic (callus: 228.51 mg kg^{-1}) compared to that of infusion extract of *M. longifolia* of Turkey (162.7 mg kg^{-1})[8]. Moreover, the gallic acid is only identified in C-glycosides plant with 17.03 mg kg^{-1}, this value is higher than those of *M. longifolia* of correspondingly Turkey and Iran (4.6 mg kg^{-1} and 2 µg g^{-1})[8,34]. Concerning the content of naringenin (flavanone), it is weak (0.28 mg kg^{-1}) in accordance to the polyphenol profile of *M. longifolia* of Hungary which has showed the lack of this compound in mixture ethanol and water extract[32].

These results could be dependent on the geographical location (environmental, harvest site) factors and the extract used. The increase of phenolic compounds content are likely related to the arid conditions (drought, low rainfall, and high radiation), characterizing Ahaggar in southern Algeria. This aridity may enhance phenolic compound synthesis as a response to harsh conditions.

Acute oral toxicity

The animal monitoring treatment by the dose of 2000 mg kg^{-1} of plant and callus infusion, showed normal appetitive and calm behaviour of animals after 4 h of treatment. No mortality was registered for 14 days. We confirm that the aqueous form and the oral administration are beneficial to preserve the healthy care of animal models.

Biological activities

Anti-inflammatory Activity

The results of anti-inflammatory activity of the plant and callus, at doses tested (1 g kg^{-1} for aqueous and 250 mg kg^{-1} for C-glycoside) extracts are reported in Table 3. Regarding the control group (physiological water 0.9%), the left paw injected volume increased up to 3 h and the difference value between left and right hind paw volume is high. The reference medical drug (aspirin, ASA) decreased significantly the inflammation at P <0.01 with a maximum value observed of 70.8%. The oral administration of aqueous extracts of both plant and callus at 1 g kg^{-1}, showed the same inhibition value of 64.05%, whereas, C-glycoside extracts revealed no significant difference comparing to the control group with respectively 20.6% (plant) and 1.3% (callus) inhibition. We suppose that the isolation of the whole constituents of plant and callus in water (aqueous extracts) are beneficial for their bioavailability. This statement was previously mentioned to be efficient for some phenolic compounds related to the extraction method[35].

Also, the infusion mode may preserve the efficiency and the activity of chemical compounds; that remark was equally noted on the possible effect of plant material preparation (cooked, stewed or boiled) on the secondary metabolites of Citrus, these authors confirm that heat treatment maintains the anti-inflammatory potency of naringenin[36].

In overall, the weak inflammation reduction showed by the butanolic extract as well as by plant and by callus trend to conclude that the callus was not efficient in this solvent. Our observations are similar to those reported that the weak values of C-glycosides diluted in butanol might be related to other compounds such as flavonoids well-known as inflammatory substances[37].

In contrast, the same percentage inhibition of aqueous plant and callus extracts, suggests the strong anti-inflammatory action of callus, this positive response of callogenic cells might be due to the activity of rosmarinic, caffeic, p-coumaric, gallic and ferulic acids. These findings are in agreement with previous observations of the anti-inflammatory role of gallic acid of *Radix sangisorbae*, thus, this compound is considered as a marker component to produce anti-inflammatory effects by inhibition of prostaglandin production in lipopolysaccharide[38].

Table 3—Anti-inflammatory activity of group using carrageenan induced mice paw edema

Materials	Groups	Dose (mg)	Paw weight (g)			% Inhibition
			Left paw	Right paw	Difference	
	I	0.5 mL	0.159±0.009	0.118±0.006	0.042±0.02	----
	II	10	0.149±0.01	0.135±0.01	0.013±0.004**	70.8
Plant	III	1 000	0.149±0.008	0.133±0.01	0.016±0.01^{**nss}	64.05
	IV	250	0.173±0.01	0.135±0.006	0.0377±0.01$^{ns\ a}$	20.6
	V	1000	0.151±0.008	0.135±0.01	0.0166±0.01^{**nss}	64.05
Callus	VI	250	0.182±0.02	0.141±0.01	0.0411±0.02$^{ns\ b}$	1.3

[Data expressed as mean± SD (n=6). One-way ANOVA, post hoc Tukey's Test, (*ns: not significant*, ***P* <0.01 relative to control group). (nss: not significant; ^{a}P <0.05, ^{b}P <0.01, relative to reference group, where Gr. I, control (physiological water 0.5 mL); Gr. II, Standard drug (Aspirin) treated; Gr. III, Plant aqueous extracts; Gr. IV, Plant C-glycoside; Gr. V, Callus aqueous extracts; and Gr. VI, Callus C-glycoside]

On the other hand, all phenolic acids revealed in our extracts are linked to anti-inflammatory responses comparing to prior investigations, such as, ferulic acid considered to be an excellent anti-inflammatory component by its possible action on the inhibition of superoxide generation in peritoneal macrophages[39].

Rocha *et al.*[40,41] have examined the anti-inflammatory effects of rosmarinic acid extract of *Rosmarinus officinalis* (Lamiaceae) on rats; it has been found that the dose of 25 mg kg^{-1} reduced paw edema by over 60%, and it exhibits a dose-response effect, suggesting that rosmarinic was the main contributor to the anti-inflammatory effect. Also, rosmarinic acid causes a substantial reduction of inflammation on both *in vitro* and *in vivo* models; its anti-inflammatory potential has been identified. This phenolic acid might be useful in the pharmacological modulation of injuries associated to inflammation[40,41].

On the other hand, the anti-inflammatory response of caffeic acid was evaluated using carrageenan induced pleurisy model and tail-flick assay in rats. The analysis of cells in the pleural exudates revealed a reduction of 92.9% for animals treated with 10 mg kg^{-1}, and inhibition of leukocyte migration to the inflammation comparing to a reduction of 79.2% in the number of total exudate cells in animals treated by 5 mg kg^{-1} of caffeic acid[42].

Pragasam *et al.*[43] have also demonstrated that p-coumaric acid influences anti-inflammatory activity in adjuvant-induced arthritic rats by showing decrease the expression of inflammatory mediator TNF-α. Hence, this phenolic acid could be considered a potential immunosuppressive factor in the treatment of autoimmune inflammatory diseases like rheumatoid arthritis.

In addition, crude methanol extract *M. longifolia* and its fractions including butanol, has been shown to reduce NO secretion in macrophages by scavenging NO and inhibiting iNOS mRNA expression, and also decrease TNFa pro-inflammatory cytokine expression, thereby suggesting that *M. longifolia* is useful in the treatment of inflammatory diseases[37]. Such anti-inflammatory response of callus could be due to its rich phenolic acids content.

Analgesic activity

The results showed that all extracts and the reference drug, paracetamol (100 mg kg^{-1}) significantly (P <0.001) reduced abdominal writhing in mice when compared to the negative control group (Table 4).

Table 4 — Results of writhing number and percentage inhibition of acetic acid-induced writhing response in mice of both plant and callus

Materials	Groups	Dose (mg)	Writhing number	Percent inhibition (%)
	I	0.5 mL	56±0.8	----
	II	100	$28\pm0.8^{***}$	50
Plant	III	1 000	$13\pm1.2^{***b}$	76.8
	IV	250	$36.50\pm9.7^{***ns}$	34.8
	V	1000	$16\pm5.8^{***a}$	71.4
Callus	VI	250	$15.75\pm4.2^{***a}$	71.9

[Data expressed as mean± SD (n=4). One-way ANOVA, post hoc Tukey's Test. $^{***}P$ <0.001 relative to control group. ns: not significant, ^{a}P <0.05, ^{b}P <0.01, relative to reference group, where Gr. I, control (physiological water 0.5 mL); Gr. II, Standard drug (Paracetamol) treated; Gr. III, Plant aqueous extracts; Gr. IV, Plant C-glycoside; Gr. V, Callus aqueous extracts; Gr. VI, Callus C-glycoside]

Higher percentages inhibition of writhing (plant; 76.8% and callus; 71.4%) were recorded by aqueous extracts at 1 g kg^{-1}comparing to paracetamol (50% at 100 mg kg^{-1}). Equally, a big difference of percentage inhibition were obtained by C-glycoside extracts (plant; 34.8% and callus; 71.9%). This suggests the positive action of callus over analgesic activity on mice as well as in aqueous state than in C-glycoside form. This analgesic potential could be associated with the contents like rosmarinic and caffeic acids[44]. Other finding suggests that the analgesic effect of rosmarinic acid is mainly due to the acetyl ester derivative from rosmarinic acid, this product has displayed a significant analgesic activity by reducing carrageenin-induced paw edema[45]. Previous works have demonstrated the anti-inflammatory and analgesic properties of caffeic acidand rosmarinic acid and thereby have potential applications in pain and inflammatory diseases[46,47]. Even if the C-glycoside callus extracts reduce the writhing induced by the acetic acid unlike, the reduction of inflammation did not seem to be affected.

Conclusion

The present results confirm that the mint, *Mentha longifolia* possess anti-inflammatory and ant-analgesic properties. Efficacy of this aromatic and medicinal plant and callus could be attributed to the present of chemical compounds, phenolic, in particular. This study suggests that adding elicitors such as yeast extract, salicylic acid or other types of polyphenols in culture media it enhances the anti-inflammatory and analgesic activities. Further, we can conclude that the callus has a better

analgesic by its bioactive components than that the adult plant.

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Conflict of Interest

Authors declare no conflicts of interest.

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