EVALUATION OF ACUTE TOXICITY AND ANTI-INFLAMMATORY ACTIVITY OF CALLUS EXTRACTS OF *PULICARIA INCISA* (LAM.) DC.

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Abstract. This study was aimed to investigate the effect of bioactive molecules of callus infusion of *Pulicaria incisa* (Lam.) DC, by the evaluation of anti-inflammatory activity and acute toxicity. 70% was the higher rate of callus obtained from young capitula cultivated on Murashige and Skoog (1962) (MS) solid medium containing 2.4D 4.10^{-5}M and KIN 5.10^{-6} M for 4 months. A Phytochemical screening based on colorimetric reactions was performed on the callus infusion material. Unlike the absence of primary components (starch, reducing sugars), secondary metabolites such as mucilage, saponins, flavonoids and anthocyanins were revealed. No sign of toxicity was observed by the application of 0.25 to 5 g/kg doses in this investigation, although, a significant anti-inflammatory response was revealed at 5 g/kg compared to that of Diclofenac®. The chromatographic analysis (HPLC) detected the presence of 3-hydroxy-4-methoxycinnamic acid the main phenolic acid produced by callus, which could be responsible of the positive anti-inflammatory reaction. These findings suggest that the callus could be an alternative source of bioactive metabolites useful in the human health as an anti-inflammatory source.
Keywords: *Pulicaria incisa (Lam.) DC., Asteraceae, Tamanrasset, callus infusion, bioactive metabolites, biological effects*

Introduction

Pulicaria incisa (Lam.) DC., (*Asteraceae*) called "Tamayout" or "Ameo" is an endemic aromatic and medicinal herb with golden yellow capitula (flowers) rich in flavonoids and phenolic compounds (Ewais et al., 2014). It is traditionally used by aboriginal people "touaregs" in southern Algeria (Tamanrasset) to treat human pains as flu, fever, coughs, diabetes, palpitations (Maiza, 2008) and also to relieve carminative and anti-inflammatory troubles (Boumaraf et al., 2016). Scientific available studies reported the presence of several metabolites which could have a role in these healing reactions such as sesquiterpene lactones (Ghouila et al., 2009), the flavonoids (Mansour et al., 1990) and the caryophyllene derivatives (San Feliciano et al., 1989). Face to the common use of this aromatic and medicinal plant (PAM) by aboriginal population and healers by its "random-harvesting" without any ecological preservation measurement and the climate changes as rainfall less, soil salinization (Rouane, 2012), the number of "plant feets" is being less and less accessible.

Note that, some of endemic plant species in this Saharan area of Algeria early known for its rich flora are currently disappeared by these problems (Nacer bey et al., 2015). To ovoid this current critical situation, tissue culture techniques are being as alternative pathway to obtaining new plant material as callus and cell suspension considered as source for

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bioactive component production (Eid and Metwally, 2017). To our knowledge, there is no publication thus on the formation of callus with the secondary metabolites contents before and after callus establishment on this Asteraceae. This manuscript presents the results of the evaluation of a therapeutic power of aqueous extract from callus 4-months by performing chemical screening followed by the determination of the LD_{50} and the anti-inflammatory activity test on mice.

Materials and methods

Plant material and callus induction

The fresh young-inflorescences of *Pulicaria incisa* Lam. (DC.) were harvested in March 2013, in natural area of Ahaggar Tamanrasset, Latitude: 22° 47′ 13″ North, Longitude: 5° 31′ 38″ East, Altitude: 1470 m), recognized according to the plant features by using the flora (Ozenda, 2004) and the book entitled The Hoggar, botanical trip (Sahki and Sahki, 2004), confirmed by the botanists of National Institute of Forest Research "INRF" of Tamanrasset (Tam). The Specimen voucher was deposited in National Herbarium of the Research laboratory of Arid Zones LRZA Herbarium for authentication (N°2-2012 Tam; PAM/LRZA/USTHB).

After isolation, natural capitula of 8 mm diameter were freshly taken, then, sterilized in Mankooza solution (0.01%) for 30 min, well rinsed, thus, immersed in mercuric chloride $HgCl_2$ (0.01%) for 10 min and rinsed with sterile distilled water 3 times of 5 min each. The capitula were, dried and cultivated on Murashige and Skoog (1962) medium supplemented with 2 mL Morel and Wetmore vitamins (1955), 3% (w/v) sucrose (30 g L^{-1}), 0.8% (w/v) agar (8 g L^{-1}) and different growth regulators combinations and concentrations (*Table 1*). The medium was adjusted to pH 5.8 and autoclaved at 120 °C for 20 min. The cultures were incubated at 27 ± 1°C in total darkness until callus induction and high multiplication. The application of hormone (growth regulators) concentrations and combinations were chosen as treatment to express the morphogenetic capacities of the explants (Chabane, 2007; Rouane, 2012).

Table 1. *Different media composition in Auxin-cytokinin concentrations tested*

Media	Auxins/Cytokinins (mole/L)				
	2.4D	**NAA**	**IAB**	**BAP**	**KIN**
Control	None				
M1	5.10^{-4}	-	-	4.10^{-6}	-
M2	3.10^{-5}	-	-	-	5.10^{-6}
M3	4.10^{-5}	-	-	-	5.10^{-6}
M4	-	5.10^{-6}	4.10^{-6}	3.10^{-4}	5.10^{-6}

2.4 D: 2.4-dichlorophenoxyacetic acid, NAA: naphthalene acetic acid, IAB: Indole butyric acid, BAP: 6-benzylaminopurine, KIN: kinetin

Preparation of infusion extracts

Two types of infusion extracts were prepared, in boiled water (for phytochemical screening analysis) and in NaCl (0.9%) for bioactivity tests (anti-inflammatory and acute toxicity).

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5 g of each, callus (fresh) and natural capitula (young flowers, air-dried and chopped) were separately soaked in 100 mL of boiled solution for 20 min, then filtered through Wathman filter paper (Cat N°40, 1440-110), the filtrate obtained was used. Note that, natural capitula were only used in phytochemical screening to determine the overall chemical composition of callus obtained by in vitro culture assays.

Animal preparation

Albino mice *Mus musculus* (20-30 g weight intervals, 10-12 weeks-old) under temperature of 22-24 °C, 50% humidity, 12 h photoperiod, feeded by pellets scheme and tap water ad libitum were chosen. The Statement of safety was approved by the ethical committee of (CRD-SAIDAL, Algiers).

Biochemical composition

Phytochemical screening

Callus and natural capitula infusions were separately treated with various solvents (*Table 2*) to detect the different bioactive components by colorimetric or precipitation reactions as shown in *Table 2* (Harborne, 1998; Bruneton, 2009).

Table 2. *Phytochemical screening of natural capitula and callus infusion*

Compounds		Chemical reagents	Colorimetric reactions
Primary metabolites	Reducing sugars	Fehling liquor	Orange precipitation
	Starch	Iode (I_2)	Purple blue
	Glucosides	H_2SO_4	Orange-red-purple
Secondary metabolites	Mucilages	Ethanol	Flaky precipitate
	Flavonoids	HCl + Magnesium chips (Mg) + Isoamelic alcohol	Orange-red
	Anthocyanins	HCl Ammonia ½	Red Blue
	Total tannins	Fe Cl_3 5%	Blue black
	Catechic tannins	Stiansy reagent (formol+HCl)	Red
	Saponins	NaOH 0.1 N / HCl 0.1 N	Foam formation
	Iridoids	HCl /heating	Blue

NaOH: sodium hydroxide, HCl: hydrogen chloride, $FeCl_3$: ferric chloride, H_2SO_4: sulfuric acid

High-performance liquid chromatography analysis (HPLC)

High-performance liquid chromatography (HPLC) was carried out with a Agilent 1100 System, quaternary pump, on-line degasser automatic injector and UV detector with bars of diodes surveyor (DAD), using Hypersil BDS-C18 column (250 × 4.6 mm, 5 μm) at 30 °C. The chromatographic data system was controlled by the mobile phase (acetic acid 0.2%/ACN) for 30 min. The solvent flow rate was 1 mL/min.

Biological activities of callus infusion

The bioactivity determination by two tests (anti-inflammatory and acute toxicity) concerns the application of callus infusion on animal models chosen.

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Acute toxicity test

The examination of acute toxicity was performed in oral gavage as prescribed by Litchfield and Wilcoxon (1949). A total of 5 mice (20-30 g) in separated cages of each group; (control group and 5 experimental groups) were fasted 18 h before experiment. An oral gavage of 0.5 mL of callus infusion was administered as follows; 0.25 g/kg, 0.5 g/kg, 1 g/kg, 2 g/kg and 5 g/kg, while the control animals were received the NaCl (0.9%). NaCl expresses the physiological water 0.9% without any risk, was used for the preparation of different extracts (control and treatments) under the same conditions. The mice were monitored from 30 min to 4 h. The body weight variation, general behavioral and toxicity symptoms were noted at least once daily for 14 days (Nana et al., 2011). The DL_{50} and the maximum dose without effect (MDE) were calculated and compared to the control batch.

Evaluation of carrageenan-induced anti-inflammatory activity

The anti-inflammatory activity of callus extracts of *Pulicaria incisa* was performed using inflammation by edema formation induced by carrageenan on hind-paw of mice (Winter et al., 1962). Four groups of 10 male mice in each (20 to 30 g) were fasted 18 h before the beginning of the experiment. A dose of 0.5 mL was orally given as follows; control group (NaCl 0.9%), experimental groups by callus infusion at 2 g/kg (I), 5 g/kg (II) and Diclofenac® at 5 mg/kg (experimental group III). 30 min after each administration, 25 µL of the 1% w/v carrageenan was injected subcutaneously into the plantar aponeurosis of the left hind paw of each mouse. The behavior and responses of all mice were observed and noted (Zhang et al., 2015). Three hours later, the animals were quickly sacrificed. The variation of paw volume of each animal was noted, then, the mean of weight (M) for each group was calculated. The following equations *(Eqs. 1–2)* were used to calculate the percentages of the edema (%E) and of edema reduction (% E.R) respectively:

$$\%\,\mathrm{E} = \frac{(\mathrm{M\ LPW} - \mathrm{M\ \ RPW})}{\mathrm{M\ \ RPW}} \times 100 \qquad \text{(Eq.1)}$$

$$\%\,\mathrm{E.R.} = \frac{(\%\ \ \mathrm{E\ PC} - \%\ \mathrm{E\ PE})}{\%\ \mathrm{E\ PC}} \times 100 \qquad \text{(Eq.2)}$$

where: % EPC: percentage of edema of the paw of the control, % EPE: percentage of edema of the paw of the experimental groups, LPW: Left Paw Weight, RPW: Right Paw Weight, M: Mean of weights.

Statistical analysis

The results obtained were analyzed using Statistica Software (6.0). Data were expressed as means ± ESM (or means ± SD), then were subjected to One way analysis of variance (ANOVA) followed by Duncan Multiple Range Test (DMRT) and differences between data means were regarded statistically significant at $P < 0.01$ and $P < 0.05$ (Duncan et al., 1977).

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Results

Callus induction

This study showed that the young capitula reacted positively onto media tested with 30 to 70% callus induction ratios; M2 (2.4D 3.10^{-5} M/KIN 5.10^{-6}M) with 60% and M3 (2.4D 410^{-5} M/KIN 5.10^{-6}M) with 70% compared to that of M4 (NAA-IAB/BAP-KIN) with 30% (see *Fig. 1, Table 3*).

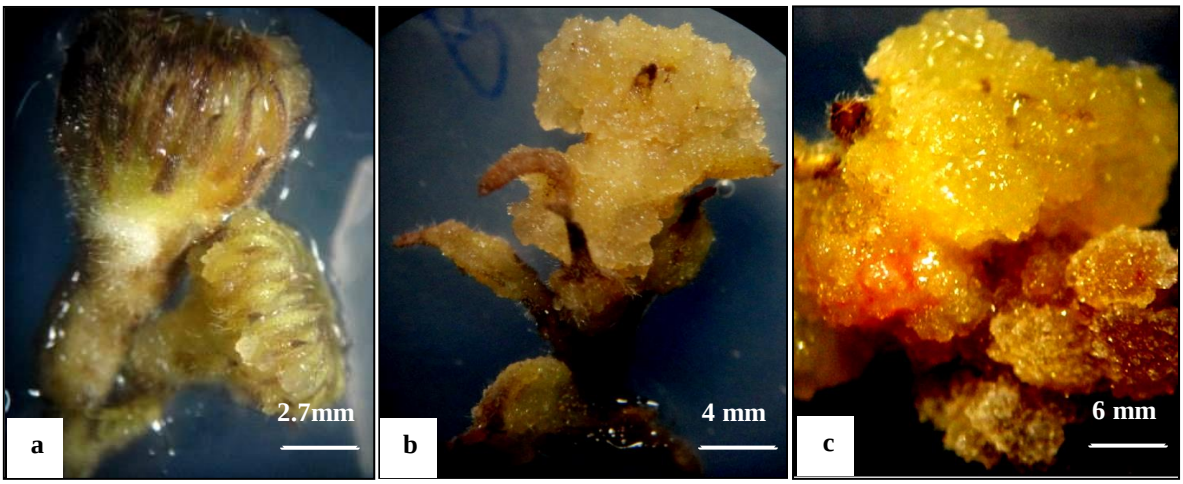

Figure 1. *Callus induction and multiplication of capitula explants of Pulicaria incisa on culture media (M 3).* ***a*** *Induction after 2 weeks,* ***b*** *friable callus formation after 4 weeks,* ***c*** *callus multiplication after 6 weeks*

Nevertheless, no callus was observed onto M1 (2.4D/BAP) even the long maintenance of explants (32 weeks). The M3 is thus, considered the most successful of callus formation in this experiment. The color and morphological aspects of calluses varied with time and the concentrations of growth regulators. All callus masses obtained were friable with variable multiplication degree (low, middle and high) from 40 to 80 mm diameter.

Table 3. *Callus induction frequency (%) from Pulicaria incisa young capitula after several weeks of culture*

Media Aux/Cyto	Call. (%)	Call. col. morph.	Degr. call. form.	W. cult.
Control	0	None	-	32
M1: 2.4D/BAP	0	None	-	32
M2: 2.4 D/KIN	60	Friable, yellowish, whitish to brown	++	2
M3: 2.4 D/KIN	70	Friable, yellowish to whitish brown	+++	2
M4: NAA, IAB/BAP, KIN	30	Friable, whitish to brown	+	6

+: poor, ++: moderate, +++: profuse, Aux/Cyto: (Auxins/Cytokinins) combinations, 2.4 D: 2.4-dichlorophenoxyacetic acid, NAA: naphthalene acetic acid, IAB: indole butyric acid, BAP: 6-benzylaminopurine, KIN: kinetin, Call. (%): Callus percentage, Call. col. morph: Callus color morphology, Degr. call. form: degree of callus formation, W. cult.: weeks of culture

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The degree of callus formation expresses the variation of number and diameter of callus mass by capitula with time; +: low with diameter (d < 40mm), ++: middle (40 mm < d < 60mm), +++: higher (60mm < d < 80mm). The most important callus mass (diameter, 80 mm) was observed on 2.4D-KIN that on media added of NAA-IAB or BAP-KIN after 4 months.

Biochemical compounds of infusion extracts

Qualitative biochemical composition

The phytochemical screening has mentioned a total absence of primary metabolites (glucosides and starch) with the presence of secondary metabolites.

Table 4 summaries the variation of phytochemical compounds of callus infusion compared to that of natural capitula, callus infusion showed the total absence (-) of primary metabolites (glucosides, reducing sugars, and starch), the presence of secondary metabolites. On the other hand, natural capitula infusion revealed the presence of glucosids, flavonoids, mucilages, tannins, cathechical tannins and prints of anthocyanins. Note that, the amount of each varied from a weak (+) to important (+++). The comparison between callus and natural capitula infusion exposed a large amount of mucilage in callus, a middle content in both, of saponins and anthocyanins with a total absence to middle amount of Glucosids, Iridoids and tannins.

Table 4. *Phytochemical composition of callus and natural capitula*

	Compounds	Callus	Natural capitula
Primary metabolites	Starch	-	-
	Reducing sugars	-	-
	Glucosides	-	++
Secondary metabolites	Mucilages	+++	+
	Saponins	++	++
	Flavonoids	+	++
	Anthocyanins	+	+
	Total Tannins	-	++
	Catechic Tannins	-	++
	Iridoids	-	-

-: total absence, +: weak amount, ++: middle amount, +++: important amount

Chromatographic analyses by HPLC

The comparison of samples tested to all standards used revealed a moderate 3-hydroxy-4-methoxycinnamic acid content, considered as the main phenolic acid in callus infusion assay with 3.159% (peak area percent) recorded at λ = 260 nm (see *Fig. 2 a, b*).

Biological activities evaluation of callus

Oral acute toxicity study and body weight variation

The weighting of all animals recorded an increase of body weight of mice in all control and assays tested with time (see *Fig. 3*). The callus infusion tested on mice

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orally at various doses (0.25 g/kg, 0.5 g/kg, 1 g/kg, 2 g/kg and 5 g/kg) did not toxic, neither mortality was observed after two weeks of application. Note that, 5 g/kg was the maximum dose without effect (MDE) compared to the control batch. Duncan Multiple Range Test (DMRT) revealed a higher significant difference of the body weight between control and all doses tested at 15 days of treatment (P*** < 0.001).

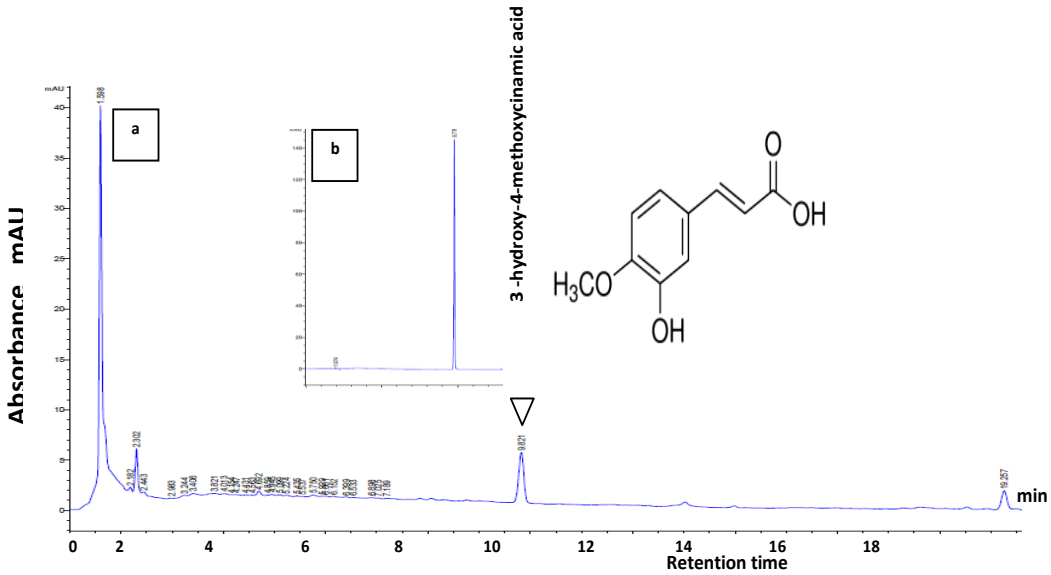

Figure 2. *HPLC chromatogram.* ***a*** *Callus infusion of Pulicaria incisa (Lam.) DC., visualized at λ = 260 nm;* ***b*** *3-hydroxy-4-methoxycinnamic acid Standard phenolic acid injected in the same conditions*

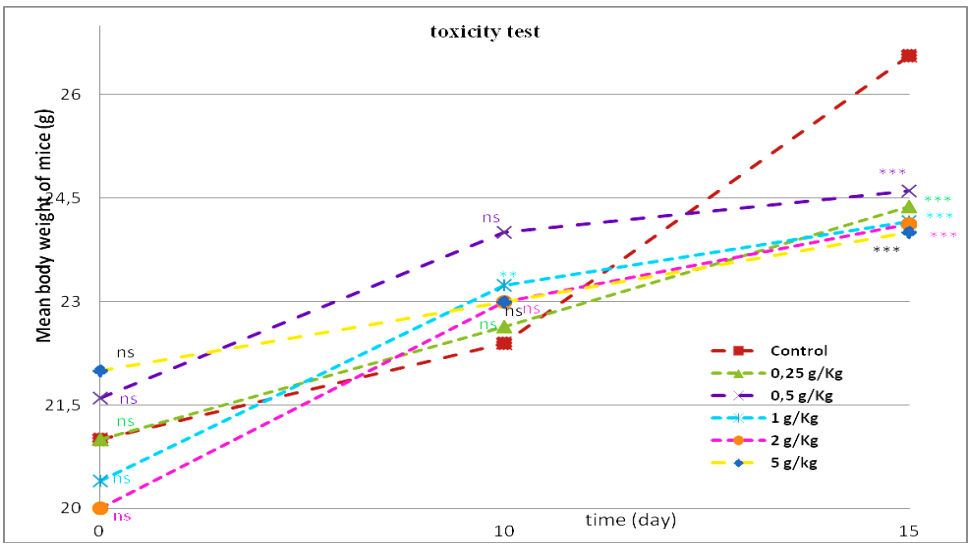

Figure 3. *Body weight variation with time of callus infusion treatment compared to the control. Means (means ± standard error). Duncan Multiple Range Test (DMRT), expressed differences of data means *** indicate a higher significant difference between the control and treatment (***P < 0.001), **P < 0.01: very significant, ns not significant*

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Anti-inflammatory activity evaluation

The anti inflammatory activity of callus infusion was investigated. As shown in *Table 5* and *Figure 4*, the monitored of weight of mice of all groups showed a decrease of the paw edema, of 37.66% (at 2 g/kg), 46.95% (at 5 g/kg) by callus infusion and 40.65% by the Diclofenac®. Duncan Multiple Range Test (DMRT) revealed a significant difference between the control compared to experimental group at 5 g/kg dose and the Diclofenac® (P* < 0.05).

Table 5. *Comparison of edema reactions (edema apparition and reduction) in mice treated with Diclofenac® (5 mg/kg) and with callus infusion*

Anti-inflammatory test, n = 10					
Doses	**Control group NaCl**	**Experimental groups**			**ANOVA one way**
		Callus infusion (I)	**Callus infusion (II)**	**Diclofenac® (III)**	
	0.9%	**2 g/kg**	**5 g/kg**	**0.005 g/kg**	
M. LPW	0.1831 ± 0.007	0.1699±0.004	0.1678±0.0041*	0.1623±0.003*	*P* 0.027 *P < 0.05
M. RPW	0.1263 ± 0.004	0.1327±0.003	0.1355±0.0035	0.1281 ±0.002	*P* 0.197 P > 0.05
% E	45 ± 0.036	28 ± 0.022	24 ± 0.021*	27 ± 0.017*	
% ER	/	37.66	46.95	40.65	

LPW: Left Paw Weight, RPW: Right Paw Weight, %E: percentage of edema, % ER: percentage of edema reduction, Mean± SEM: M Mean; ± SEM: standard error of means, n = 10: total mice in group tested, ®: Reference, *P < 0.05: compared to control, was considered significant by the Duncan Multiple Range Test (DMRT)

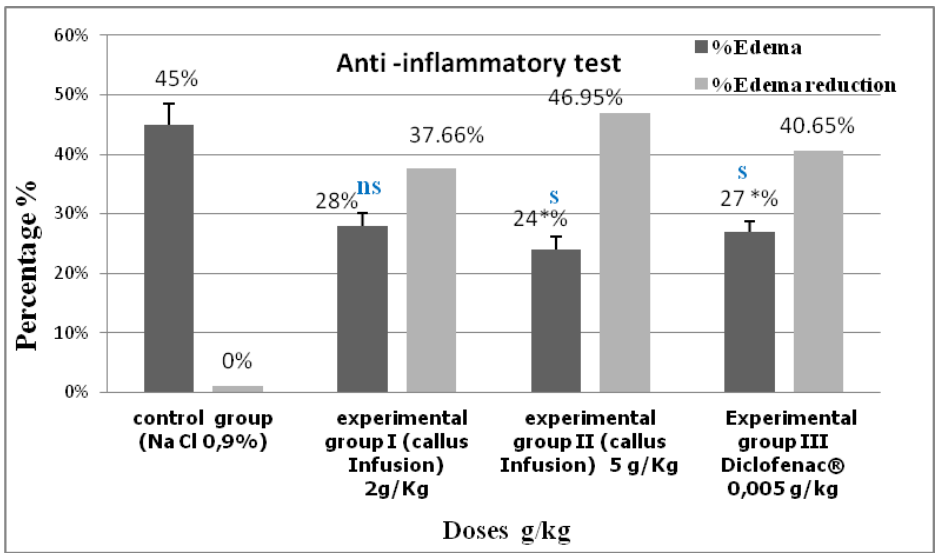

Figure 4. *Anti-inflammatory activity evaluation (%, mean ± SEM) of callus infusion (2 and 5 g/kg) compared to Diclofenac® (5 mg/kg) and control treatment. Means (means ± standard error). The values of Duncan Multiple Range Test (DMRT), expressed with a letter "s" a significant difference between the control compared to experimental group at 5 g/kg dose and the reference (Diclofenac®), with P* < 0.05; ns: not significant*

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Discussion

The influence of different concentrations and combinations of growth regulators on the induction of callus from capitula of *Pulicaria incisa* showed a better response in the medium supplemented with 2,4D and KIN. 70% was the higher callus rate obtained onto 2.4D 4.10^{-5} M and KIN 5.10^{-6}M. The same observations have been noted by Gupta et al. (2015) and Farvardin et al. (2017) who confirmed the effectiveness of 2.4D/KIN in vitro conditions by the best induction and proliferation of callus masses in dicotyledonous plants.

However, capitula cultivated on 2.4D/BAP, did not display any change in their morphological aspects except the total necrotic reaction due to the release of phenolic compounds (Ozyigit et al., 2007); which have generally a negative effect by the inhibition of enzyme functions and the inactivation of explants growth leading browning and darkening of the media (Arnaldos et al., 2001).

Because, the weak amount of anthocyanins in callus is similar to that of capitula, this suggests that dark has no influence upon action of their biogenesis, thus, it is advised to carry out further experiments by exposure of callus under light to boost the biosynthesis process. The same suggestions were previously supposed by Mathur et al. (2010) on callus line of *Panax sikkimensis* Ban. (*Araliaceae*). Also the total absence of starch and reducing sugars indicates that the plant do not synthesize or store starch. That's in agreement with Benhouhou (2005) regarding the presence of "Inulin" in *Pulicaria incisa* Inuleae tribe. However, the highest production of mucilage in callus could be a secondary energy reserve (Langenheim, 2003). Currently, Gupta et al. (2015) advise to harmonize the combinations of growth regulators to ensure the high production of mucilage under in vitro conditions. Further experiments are useful to run cultures under light conditions for the possible high secondary metabolites amounts production.

Regarding the HPLC analysis, 3-Hydroxy-4-methoxycinnamic acid, was appeared to be the main phenolic acid in callus infusion, this result is consistent with the findings of Arafa et al. (2015) and Azeez et al. (2017). These authors have reported that it is the major compound of callus produced under in vitro conditions.

In the current study, the weighting of animals recorded an increase of body weight of mice in all control and assays tested with time; it was expressed by the moderate gaining of weight. Conversely, Jahn and Günzel (1997), also Saad et al. (2016) described the toxicity sign by the weight loss. We consider that, saponins, mucilages, anthocyanins and flavonoids of callus are without any toxic prints on mice (Avachat et al., 2011; Du et al., 2015; Muthukumaran et al., 2017). Note that Hammiche and Maiza (2006) have not scored any toxic effect of *Pulicaria incisa* in the field findings.

The moderate anti-inflammatory activity of the callus infusion registered, could be explained by the presence of 3-hydroxy-4-methoxycinnamic isoferulic acid detected at 260 nm). Our results are in agreement with reports of Shiraki et al. (1998) and Kim et al. (2012), who displayed that the 3-hydroxy-4-methoxycinnamic acid has both antipyretic and anti-inflammatory activities by suppressive of interleukin-alpha compounds production. In addition, the calm state of mice during the experiments was certainly due to the higher amount of mucilage in callus, the polysaccharides, which has softening and anti-inflammatory activities, reducing inflammation (Morrow, 1998; Sindhu et al., 2012). Also, the mucilage richness of callus infusion suggests the soothe effect of inflamed tissues. We suggest that the flavonoids and anthocyanins content in the callus could apply an anti-inflammatory effect. Wiseman et al. (2001) and O'Leary et al. (2004) explained that flavonoids exert multi-level anti-inflammatory properties by

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the inhibition of the production of inflammatory cytokines. In parallel, Larrosa et al. (2010) and Havsteen (2002) have reported the synergetic important role of flavonoids and anthocyanins in the protection of veins and capillaries. Moreover, Ez Zoubi et al. (2016) underlined the significant anti-inflammatory effects of flavonoids and mucilage fractions with time on edema especially on cycloxygenase activity (COX) at the later phase by inhibition. We suppose that saponins of callus infusion act as inhibitors of prostaglandins to increase the anti-inflammatory response.

This study highlighted the successful callus induction using capitula explants and culture conditions chosen, this new material (calluses) provide a higher production of mucilage comparing to mother plant. This overview opens the minds to their use as cell cultures for the production of these active components.

These findings have also successfully showed the effective therapeutic potential of callus to consider in further biological activities. Indeed, the application of callus infusion at (2 and 5 g/kg) on animal models has revealed an interesting anti-inflammatory effect comparing to Diclofenac®, due to the production of 3-hydroxy-4-methoxycinnamic acid and other components.

Conclusion

This study reports for the first time the callus formation of *Pulicaria incisa* and evaluation of the anti-inflammatory activity due to its biological composition. Based on our results, we have successfully showed an interactive relationship between the pharmacology and the plant biotechnology which could be applying into the large-scale active principles production in the future. In addition, these findings suggest that the callus extracts has a positive oral safety, which is non-toxic, may be useful in human health by anti-inflammatory effects. These effects could be ascribed to phenolic compounds such as 3-hydroxy-4-methoxycinnamic as anti-inflammatory source with markedly reduce edema. Thus, future studies are needed to induce the production of high secondary metabolites amounts in calluses mass, by varying elicitors and medium composition, then, should examine the major components isolated individually after identification by HPCL. The application of this process on other endemic medicinal plants could be more advantageous for metabolites production and brittle plant species preservation independently of current ecological conditions.

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