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Evaluation of the *Halopitys incurvus* algae essential oil as a wound-healing candidate: An animal study

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Abstract

The use of seaweed in medicine has been known since antiquity but remains limited. The skin is an organthat has essential roles for the good functioning of our body but also in the image we give to others. This study lies within the scope of the valorization of essential oil of *Halopitys incurvus* alga field as antioxidants and healing. The methods applied to measure the antioxidant activity were trapping of the free radicals by using the 1,1'-diphenyl-2-picrylhydrazyl (DPPH°); ferric reducing antioxidant power (FRAP): hydroxyl radical scavenging; production of substances reacting with thiobarbituric acid (TBARS) and the estimation of total phenolic content. For evaluation the healing activity we used induced surgical wounds in Wistar rats. The results obtained show that the essential oil of the red algae *Halopitys incurvus* has a very significant antioxidant activity with all the tests used, DPPH, FRAP, Hydroxyl radical, TBARS with the respective IC_{50} values 106.05 ± 0.41, 264.8 ± 0.28, 208.51 ± 0.85, 100 ± 91(µg/ml). Also we discovered a very important healing capacity on the wounds produced on the Wistar rats. The results obtained confirm a correlation between the phytochemical study, the antioxidant activity and the healing capacity that demonstrate the essential oil of the red algae *Halopitys incurvus* constitutes a reservoir of components with a very interesting effect in the healing process.

Keyword: *Halopitys incurvus*, essential oil, phytochemical study, antioxidant activity and healing capacity.

1. Introduction

The skin indispensable to life, protective covering of the body, is a physical barrier protecting all organs and tissues from external aggressions **[21]**. The skin is considered as the most extensive but also the most voluminous organ of the human body with a surface close to $2m^2$ **[21]**, for about 16% of the total weight of the individual. Disruption of skin integrity can occur in several contexts: surgeries, burns, radiation, cuts, tears, scrapes, abrasions, chafing, pinching and pressures.

Wounds are an extremely common reason for consultation in emergency departments. This type of seemingly trivial accident can pose complex therapeutic problems. Skin wound healing is a coordinated dynamic process that involves cell multiplication, active cell migration and extracellular matrix production **[3,4]**. Wound healing is a natural, multifactorial and dynamic pathophysiological process aimed at restoring the integrity and functionality of damaged tissues **[19,20]**. There are two healing processes: regeneration and repair **[12]**. Despite the regenerative potential visible in the fetal stage, most wounds in adult mammals heal by the repair mode **[9] [18,22]**.

Essential oils used since antiquity in an empirical way as the most practical therapeutic agent against various ailments arouse more and more the interest of scientists. They are the subject of active study in the world for their possible use for the preservation of foods against oxidation and as remedies against infections **[29].**

Seaweed is an important source of bioactive natural substances, which are synthesized by metabolic pathways different from those observed in terrestrial environments **[14]**. The *Halopitys incurvus* red algae include numerous compounds such as minerals, polysaccharides, derived amino acids, fatty acids, sterols, carotenoids and phenolic compounds such as flavonoid and condensed tannin by a study already done in our laboratory **[14,27,16]**. In addition, *Halopitys incurvus* algae have a very significant anti-free radical effect which has been proven by the reducing activity of DPPH.

The objective of the present study is to show that the essential oil of *Halopitys incurvus* seaweed has a very significant antioxidant and healing activity and to confirm the correlation between the presence of phenolic compounds and the antioxidant and healing activities.

2. Experimental

2.1. Plant material

The plant material is the essential oil of the red alga *Halopitys incurvus.*

- ***Halopitys incurvus*** It is a locally abundant perennial algae all year round, collected by hand-picking from the coast of El Jadida Morocco (33° 33°16’09’’N, 8°30’ 8°45’W). The algae **(Fig.1)** were cleaned, washed in distilled water, air dried at room temperature, powdered and stored. **[15]**
- **Essential oil preparation**

The *Halopitys incurvus* alga is cut into very fine parts and subjected to hydrodistillation using the Clevenger-type extraction device **(Fig.2)**. Hydrodistillation is based on the power of water vapor to transport essential oils **[8]**.

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Figure 1: *Halopitys incurvus*

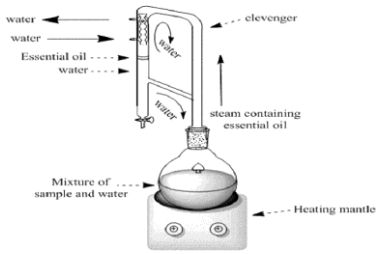

Figure 2 : Hydrodistillation set-up

2.1 Thin layer chromatography for phytochemical characterizations

Thin layer chromatography (TLC) is a liquid phase chromatography technique. To perform the separation, a small amount of the solution to be analyzed is placed on the edge of a (TLC) plate. Phytochemical characterization was performed by thin layer chromatography (TLC) for flavonoids, tannins, alkaloids and sterols/triterpenes. Aluminum chloride was used for the detection of flavonoids, ferric chloride for the detection of tannins, Dragendorff reagent for the identification of alkaloids and acetic anhydride associated with chloroform then sulfuric acid for the characterization of sterols/triterpenes.

2.2. Evaluation of total phenolic contents

Total phenolic contents of essential oil of *Halopitys incurvus* were measured using Folin- Ciocalteu method as described by Taga et al. **[30]**. The absorbance was measured at 750nm and total phenolic content was calculated using gallic acid curve and expressed as gallic acid equivalent per gram of extract (mg GAE/g).

2.3. Determination of flavonoid contents

Flavonoids of essential oil of *Halopitys incurvus* were estimated using the method of Dehpeur et al. **[11]**. The absorbance of the reaction mixture was measured at 415nm with a double beam spectrophotometer (Perkin Elmer). The flavonoid contents were calculated, from a calibration curve, as milligram quercetin equivalent per gram of extract (mg QE/g).

2.4. Determination of condensed tannin contents

The amounts of tannins of essential oil of *Halopitys incurvus* were estimated using the method described by Price et al. **[25]**. Condensed tannins were determined by vanillin under acidic conditions. The absorbance of the reaction mixture was measured at 500nm. The results were expressed as milligram catechin equivalents per gram of extract (mg CE/g).

2.5. Evaluation of free radical scavenging activity by DPPH method

Antioxidants react with the stable free radical DPPH (deep violet color) and convert it to 1,1-diphenyl-2-picrylhydrazine (DPPH-H) with discoloration (yellow). The free radical scavenging activity of Essential oil of *Halopitys incurvus* was measured as described by Bounatirou et al. **[6]**. The absorbance was measured at 517nm and the synthetic antioxidant BHT was used as standard. For each sample, the radical scavenging activity was calculated as a DPPH percentage inhibition:

Inhibition (%) = 100 x (A0 −A1) /A0.,

Where : A0: Absorbance of control, A1: Absorbance of sample.

2.6. Ferric reducing antioxidant power (FRAP)

Reducing power was measured by the direct reduction of Fe^{3+} to Fe^{2+} and was determined by measuring absorbance resulted from the formation of the Perl's Prussian blue complex following the addition of excess ferric ions (Fe^{3+}). Hence, the ferric reducing antioxidant power (FRAP) method **[24]**, the absorbance was measured at 700nm. The increased absorbance of the reaction mixture indicates greater reduction capability.

2.7. Hydroxyl radical scavenging

The trapping activity of the hydroxyl radicals was measured by the Fenton reaction. This method recommended by Bailey and al. **[4]** with some modification. The ferrous ion forms a tri-phenanthroline complex of orange red color which absorbs at maximum 508-510nm. This concept has been used for the determination of OH° in Essential oil of *Halopitys incurvus*. The same experiment was carried out with a solution of a standard antioxidant (BHT). The percentage of inhibition of hydroxyl radicals is calculated by the following formula:

% inhibition = (A test/A blank) x100

2.8 Production of substances reacting with thiobarbituric acid (TBARS)

Inhibition of linolenic acid oxidation, in the presence of *Halopitys incurvus* essential oil, was followed by the test of the ormation of TBARS **[23].** 1 ml of a 10^{-7} M linolenic acid emulsion in PBS (10^{7}M, pH: 7,4) and Tween (20) at 0,1% (V/V), were added to the essential oil to the final concentration of 10 to 100 µg/ml. The oxidation initiated by the addition of a solution, freshly prepared, of copper sulphate at the final concentration of 40µM. After incubation at 37°C, for 3h, the reaction is stopped by cooling on ice and by the addition of 10µl of EDTA solution (20 mM). TBARS were identified by adding successive samples of 1ml of 20% trichloroacetic acid solution (pH: 3,5), and 1ml of 0.78% thiobarbituric acid solution. After a second incubation at 95°C for 45 min, the samples are cooled to room temperature and the TBARS are extracted into n-butanol. The control includes the same reagents and solvents apart from the essential oil and undergoes the same treatments. BHT is used as standard antioxidant (50µg/ml).

2.8. Wound healing activity of essential oil

➢ **Animal material**

The animal material consisted of 9 male albino rats of Wistar strain from the Laboratory of Animal Physiology of the University ChoaibDoukkali (El Jadida). Before being used, the animals were acclimatized for 3 days. The animals were 12 weeks old and weighed between 150 and 200g. They had free access to water and food, were kept in cages and were placed in ambient temperature conditions (25°±1°C) with normal day and night alternation (12 hours of light and darkness).

➢ **In *vivo* healing test**

The wounds were made after the rats were anesthetized with ether then; they were placed prone on the bench. They were held there with transparent tape at the ends of the upper and lower limbs. The hair in their cervical regions was shaved with a razor blade, taking care to avoid skin lesions. The skin was then disinfected with 70° surgical alcohol **[2]**. Before excision, the depth of anesthesia was evaluated by the disappearance of various reflexes, the disappearance of voluntary movements, as well as the disappearance of responses to painful stimulation. The wounds were created using a sterile scalpel blade. These incisions wound surfaces measure 1.5 cm in the dorsal scapula region of each rat. This study was performed by essential oil of *Halopitys incurvus*.

3 batches of 3 rats, each placed in individual cages were respectively treated:

- **Batches 1:** untreated rats (negative control).
- **Batches 2:** rats treated with Madecassol 1% (healing medication).
- **Batches 3:** rats treated with Essential oil of *Halopitys incurvu*s algae.

Treatment and wound diameter measurements were taken every other day for 15 days using a graduated ruler. All animals were monitored regularly until the wounds healed completely.

The percentage of wound shrinkage or contraction was calculated using the following formula:

$$\textbf{Percentage of contraction} = \frac{\text{Initial wound area - Healed wound surface}}{\text{Initial wound area}} \times 100$$

2.9. Statistical analyses

The experiments were carried out in triplicate. The results are given as mean ± standard deviation (SD). The statistical analysis was performed using the Software Package for Social Sciences (SPSS, version 20.0, IBM Inc, USA). All tests were considered to be statistically significant at ($P<0,05$).

3. Results

3.1. Phytochemical characterization

The results of the phytochemical characterization of essential oil by the method of Bassène **[5]** revealed the presence of flavonoids, tannins, alkaloids and triterpenes**.**

Evaluation of phenolic compounds

The results of polyphenols contents showed that essential oil of *Halopitys incurvus* revealed a high level of the total phenolic compounds by a value of 34.31±0.24 mg GAE/g

The results of the quantitative study of the flavonoid contents of essential oil of *Halopitys incurvus* show the high content by a value of 19.42±0.82mg QE/g of essential oil. Also, essential oil of the seaweed *Halopitys incurvus* was marked by its high content of condensed tannins with a value of 14.63±0.71mg CE/g essential oil.

3.2. DPPH radical-scavenging activity

The method is widely used as a fast, reliable and reproducible parameter for investigating in vitro antioxidant activity of essential oil. It is based on the reduction of the alcoholic solution of DPPH in the presence of a hydrogen donor antioxidant due to the formation of the non-radical form (DPPH-H). According to the results obtained by the DPPH test, essential oil of *Halopitys incurvus* seaweed shows significant antioxidant activity with IC_{50} value of 106.05±0.41µg/ml (**Fig. 4**). The DPPH test shows a very high percentage of inhibition of 91% in a concentration of 476µg/ml **(Fig. 3).**

3.3 Ferric reducing antioxidant power (FRAP).

The ferric reducing antioxidant power (FRAP) mechanism is based on electron transfer rather a hydrogen atom transfer. The results match the Ferric reducing antioxidant power test shows significant antioxidant activity with IC_{50} value of 264.8±0.28µg/ml (**Fig. 4**).

3.3 Hydroxyl radical scavenging activity

This assay has been used for a long time for quantitative measurement of iron in various samples **[25].** It is known that if hydrogen peroxide is added to the tube before addition of 1.10-phenanthroline, then H_2O_2 will oxidize all the ferrous ion to ferric ion which is incapable of forming red-orange complex with 1.10-phenanthroline and a sharp reduction in A510 can be seen. This concept has been exploited for determination of H_2O_2 in the samples **[13,7]**.

Regarding the test of Hydroxyl radical scavenging activity, values show significant antioxidant activity with IC_{50} value of 208.51±0.85µg/ml **(Fig. 4).** Essential oil of *Halopitys incurvus* seaweed shows a very high percentage of inhibition of 99% in a concentration of 660µg/ml **(Fig. 3).**

3.3 Production of substances reacting with thiobarbituric acid (TBARS)

The thiobarbituric acid reactive substances (TBARS) assay has been used as a technique for measuring the auto-oxidation of foods and as an experimental method for assessing lipid peroxidation **[26].** The assay was used to measure the degree of lipid peroxidation in a lipid-rich substrate; MDA is a secondary product of lipid peroxidation **[26].** We used the TBARS formation technique during the oxidation of linolenic acid, instead of linoleic acid, is based on the fact that MDA (TBARS) would be formed only from fatty acids comprising at least three double bonds **[1]**.

According to the results obtained by production of substances reacting with thiobarbituric acid test, essential oil of *Halopitys incurvus* seaweed shows significant antioxidant activity by value of IC_{50} of 100±0,91µg/ml (**Fig. 4**). The maximum percentage of inhibition varies between 95% and 99% with low concentrations (200 and 250µg/ml) (**Fig. 3**), which shows a very important antioxidant activity of essential oil of the algae *Halopitys incurvus.*

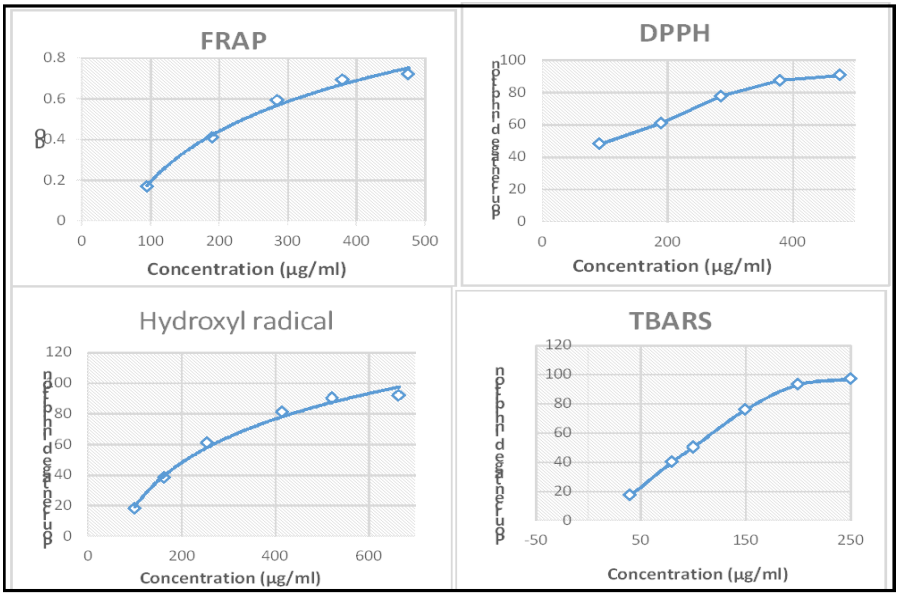

Figure 3: % of inhibition of FRAP, DPPH, Hydroxyl radical and TBARS of essential oil of *Halopitys incurvus* algae.

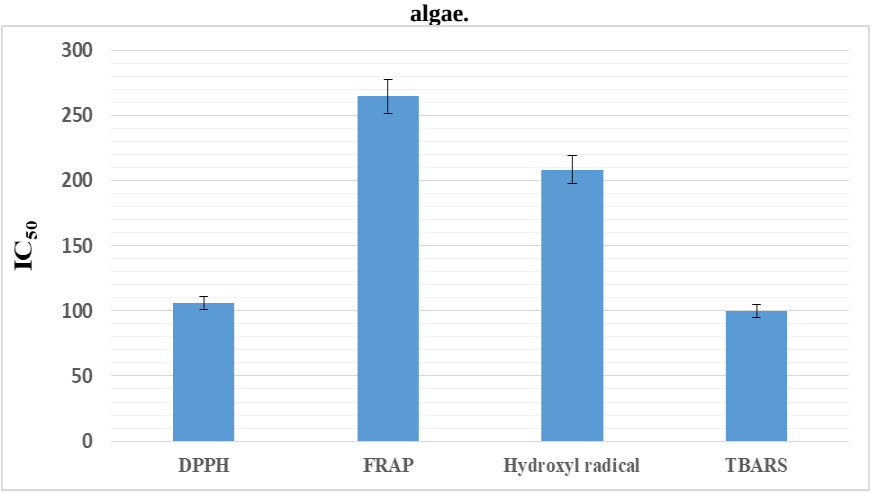

Figure 4: Evaluation of the antioxidant activity in the DPPH, FRAP, Hydroxyl radical, TBARS assay (IC_{50}) of essential oil of *Halopitys incurvus.*

3.3 In *vivo* healing test

For the treatment of wounds, traditional health practitioners mainly use plants in powder form. However decoctions, latexes, and ointments are also encountered **[10]**. The leaves of some species are used as a dressing. The use of essential oil of *Halopitys incurvus* algae for wound treatment is the purpose of this work.

In the negative control batch, the average wound surfaces between the first day and the 4th day show no decrease. From the 5th day we notice an evolution of the percentage of reduction of the surface of the wounds in the form of a slope going up to 80% on the 10th day with an average area of 0.3 cm. Between the 10th day and the 14th day, the values of the percentage reduction in the surface of the wounds show an average difference in healing with a value between 80% and 93.33%, on the 14th day with an average surface of 0,1 cm. The healing period of the rats of the negative control group is 15 days with a significant ($P<0.001$) compared to the first day.

During the first 3 days the healing does not show any decrease in the batch corresponding to the rats treated with Madecassol. From the 4th day we notice an evolution of the percentage of reduction of the wounds surface in the form of a slope going up to 66,66% on the 6th day with an average area of 0.5 cm **(Fig. 6)**. Between the 8th day and the 9th day, the values of the percentage reduction of the burnt surface do not show any remarkable difference, they form a plateau which justifies the stability of the healing of a value of 80% on the 9th day with an average surface of 0.3cm the healing period of the rats of the positive control group is 11 days with a significant ($P<0.001$) compared to the first day.

Table 1: Average wound surfaces in cm of 3 batches during the experimental period.

Days	Surface (cm) of the wounds of the rats observed over a period of 15 days		
	Negative control	**Positive control**	***Halopitys incurvus* essential oil**
D1	1.5±0	1.5±0	1.5±0
D2	1.5±0	1.5±0	1.4 ±0
D3	1.5±0	1.5±0	1.4±0
D4	1.5±0	1.4 ±0.06	1.2±0,027
D5	1±0.043	0.8±0.012	0.8±0,045
D6	0.8±0.038	0.5±0.01	0.4±0,022
D7	0.7±0.061	0.5 ±0	0.3±0,011
D8	0.5±0.042	0.3±0.022	0.1±0,018
D9	0.4±0.019	0.3±0.015	0.1±0
D10	0,3±0,014	0.2 ±0	**0** ±0
D11	0.2±0.013	**0**±0	**0** ±0
D12	0.2 ±0	**0**±0	**0** ±0
D13	0.1±0.071	**0**±0	**0** ±0
D14	0.1 ±0	**0**±0	**0** ±0
D15	**0** ±0	**0**±0	**0** ±0

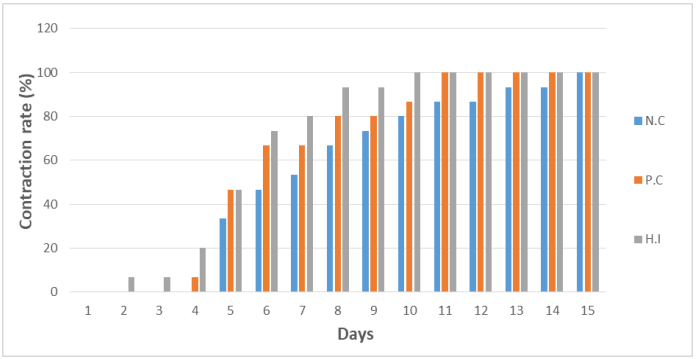

Figure 5: The plot of percentage reduction in wound area of 3 lots as a function of time.
(NC: Negative control; PC: Positive control; HI: essential oil ***Halopitys incurvus*)**

Regarding batch 3 the rat are treated with essential oil of *Halopitys incurvus* algae **(Fig.5)**. The average surfaces of the wounds from the 2nd day we note an evolution of the percentage of reduction until the 9th day with a value of 93.33%. The healing period of these rats is 10 days **(Fig. 6)** with a significant ($P<0.001$) compared to the first day.

4. Discussion

The body uses antioxidants to neutralize potentially harmful free radicals, but when there is an imbalance between the production of free radicals and the body's ability to neutralize them, the free radicals begin to damage proteins, DNA and cell membranes during a process called oxidative stress. Oxidative stress can cause premature aging: visible signs of aging can start to appear, such as fine lines and wrinkles, and skin appears dull and tired before age. Essential oil of *Halopitys incurvus* seaweed, rich in polyphenols such as flavonoids and condensed tannins, confirms the results already done on the extract of the same seaweed **[14].**

The ferric reducing antioxidant power (FRAP) mechanism is based on electron transfer rather than hydrogen atom transfer. The reducing power of *Halopitys incurvus* algae is probably due to the presence of grouping hydroxyl in phenolic compounds **[18]**, which can serve as an electron donor. Therefore, the antioxidants are considered reducers and oxidant inhibitors **[12]**. Some previous studies have also shown that the reducing power of a compound can serve as a significant indicator of its antioxidant activity potential **[32,17]**. According to the tests DPPH, FRAP, Hydroxyl radical scavenging and TBARS it was found that the red algae *Halopitys incurvus* has a very significant antioxidant activity by respective IC_{50} values 106.05±0.41µg/ml, 264.8±0.28µg/ml, 208.51±0.85µg/ml, 100±0.91µg/ml in the oil essential.

The presence of polyphenolic compound in the methanolic extract of essential oil of *Halopitys incurvus* algae could explain the healing effect observed. There are studies that have shown that the walnut extract of *A. catechu* (or betel nut) promotes healing thanks to the presence of polyphenols **[31]**.

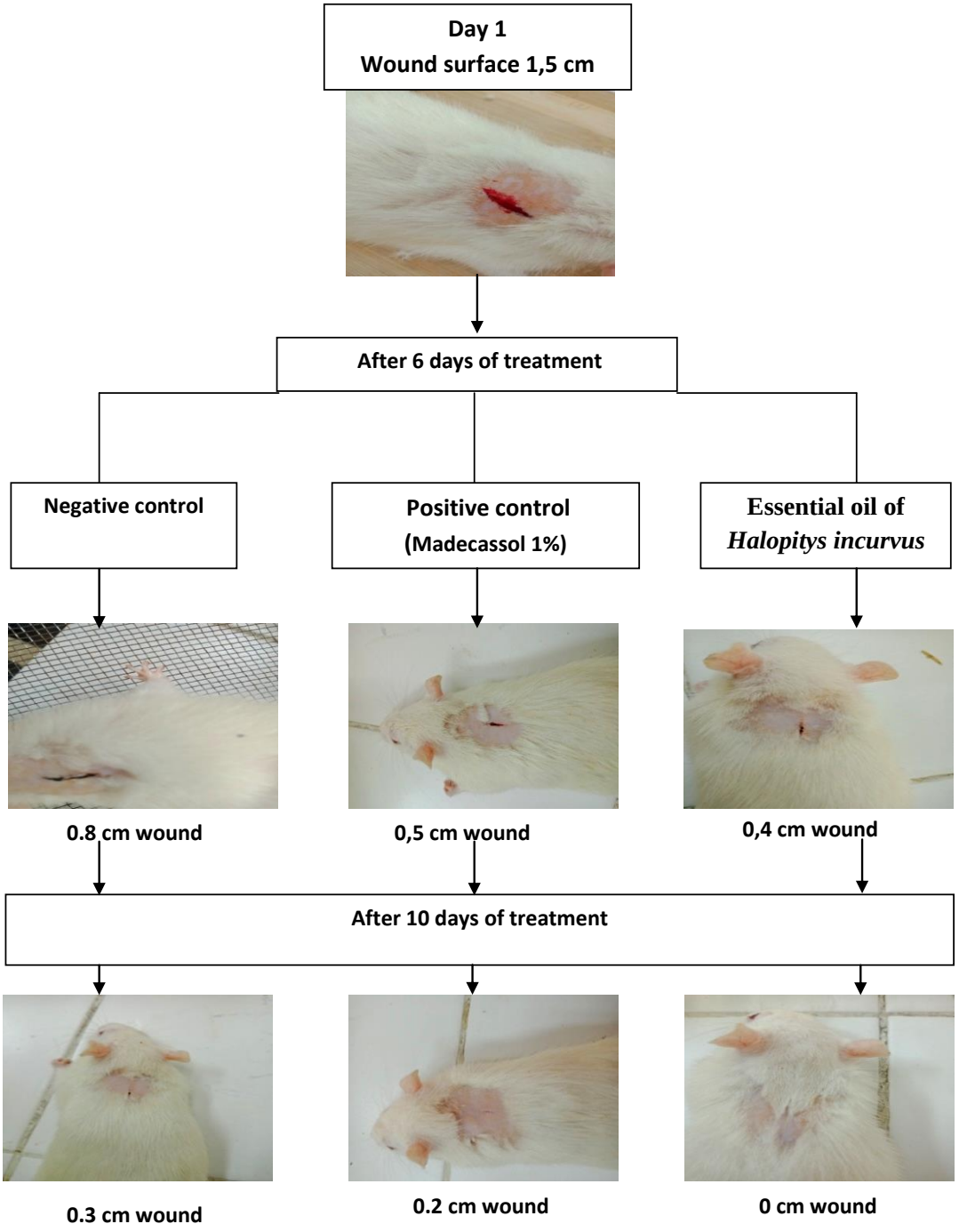

Figure 6: Evaluation of the healing activity of a 1.5 cm wound in Rats treated with essential oil of *Halopitys incurvus* algae.

Conclusion

This work aims to evaluate the antioxidant activity in vitro and the healing activity in vivo of the essential oil of Halopitys incurvus algae from the region of El Jadida (Sidi Bouzid). The first step, which consists in evaluating the antioxidant activity by four tests: DPPH, FRAP, OH° and TBARS, showed a significant antioxidant activity of the essential oil of Halopitys incurvus algae. The second step, which consists of the evaluation of the healing activity by in vivo test, revealed an interesting healing capacity. The results obtained in this present work confirm a correlation between the phytochemical study, the antioxidant activity and the healing activity and demonstrate that the essential oil of the red alga Halopitys incurvus is an active oil which could have an application in the healing process.

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Conflict of interest
The authorsdeclare no conflict of interest.

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