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Wound healing efficacy and steroid isolation from MeOH extract

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Abstract— *Achatina fulica* is a gastropod, located in the eastern and northeastern region of Madagascar, belonging to the Achatinadae family. It secretes a liquid with strong healing and curative activity which is used in traditional Malagasy medicine. Extraction and isolation by column chromatographic method, using silica and sephadex LH20 gives us metabolites belonging to the steroid class. NMR and LC-MS analyses allowed us to understand the structure of a steroid with the gross formula $C_{29}H_{50}O$. The bioassay on methanolic extract and slime affirmed that the slime of this snail is more active with a healing rate of 97.334 ± 2.053% against 72.058 ± 5.573% for the control lot (lot 4). The methanolic extract at 5% and 10% compared to vaseline is active only during the initial and repair phases.

Index Terms— *Achatina fulica*, Ischemia, Healing, Necrosis, NMR, 17-(5-ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-o.

1 Introduction

Throughout history, mollusks have provided a wide range of resources to humanity, including food, shellfish, dyes and medicines. Despite the presence of a shell, mollusks are soft, making them vulnerable to predators. They colonize marine, freshwater and terrestrial environments. Given their morphological characteristics, snails belong to the gastropod group; members of this class have a twisted visceral mass with or without shell. In many cultures, shell gastropods are considered a delicacy but also a healthy food. They are also the source of a series of traditional natural remedies (Herbert et al., 2003). However, they often live in bacteria-rich habitats such as soil or leaves (De Souza et al., 2015). As a result, they have more metabolites of interest rich in minerals and organic matter.

Recently, numerous observations have shown that the main human health problems affect the external part of their body such as spots and wounds on the skin. To solve this problem, this study will be based on the medical and cosmetic benefits of a particular snail: *Achatina fulica*.

In most cases, little scientific research has been undertaken to justify the human health benefits of these groups of animals. But currently, there is a strong increase in research on the biological activity of snail extracts as well as secondary metabolites (Nys et al., 2006). Indeed, several drugs exist on the market but their activities are slow or limited and even cause side effects. There is no effective drug, which is why the search for a new remedy of plant or animal origin is a topical topic.

The species *Helix aspersa* (small grey), which are snails of the family Helicidae distributed in Europe (France: Atlantic coast, Belgium, Catalonia...), in the countries of the Mediterranean, North Africa and North America, was a subject of study by some researchers. They have shown that the slime of this species has powerful healing and regenerating properties thanks to natural Allantoin. The bioavailability of the latter is much higher than chemical Allantoin (Liu et al., 2020), (Tzeng et al., 2022). However, for *Achatina fulica*, few researchers had not devoted more in-depth studies to prove the medical benefits that this snail can bring. However, the traditional use of some people of *A. fulica* slime on burns proves that there is a particular healing activity. The purpose of this article is to conduct a chemical investigation of *A. fulica*, to establish biological studies on *A. fulica* slime and to isolate the metabolites responsible for the healing activity in its extracts.

2 Materials and methods

2.1 Description of the study area

The Institut Pasteur in Madagascar discovered the presence of the species *Achantina fulica* in Madagascar at Diego Suarez in 1982 by J. Breuil and P. Coulanges. However, this species also exists in the province of Tamatave. The samples of *Achantina fulica* were collected in October 2017 in the district of Mananara-Nord, Analanjirofo region. It is located in geographical area 49, 76573833 S and -16, 16248183 E. This species prefers humidity and warm temperatures. In this region, populations find that the snail is a factor of destruction of vegetable crops despite their necessity. The populations of the east coast recognize this snail under the vernacular name "A*nkôra*". This species sometimes lives in dark and humid places.

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2.2 Extraction, fractionation and purification

Thus, samples collected are analyzed in the OCH-MS laboratory. The first thing that was done was extraction, fractionation, and purification. The snails were crushed with their shells using a pestle, chisel and mortar to facilitate contact with the solvent. Immediately after the sampling, 880.7 g of *Achatina fulica* were ground and extracted first with hexane, at room temperature and under mechanical agitation 500t/min until the crushed snail ran out for 72h. The solvent was renewed every 24 hours. After filtration and evaporation, a mass of fat-rich extract is obtained. The marc was then recovered after drying and reused for a second cold extraction. For the second extraction two systems were used: maceration in a dichloromethane-methanol mixture (50.50 v/v) and maceration in an EtOH-H_2O mixture (80/20 v/v) (Figure 8) to avoid the use of toxic solvents such as (DCM, MeOH) and compare the best extract yield. So we recorded the yield of the extracts obtained due to the ratio between the initial mass and the mass of the extracts obtained.
The 50/50 DCM/MeOH extract after evaporation was solubilized in MeOH/Water in a ratio of 80/20. The solid-liquid mixture was agitated until the extract was completely miscible. The hydro-methanol solution was poured into a separating funnel and extracted three times with dichloromethane (3 X 50ml DCM). After settling, the lower phase was recovered, that is, the extract with dichloromethane, then evaporated. In the second phase three times fifty milliliters of solvent ethylacetate (3 X 50 ml of AcOET) were introduced and stirred decanted recovering the upper phase. The upper phase is the ethyl acetate extract and after evaporation the acetate extract is obtained. Next, an ethylacetate extraction was performed using the same procedures as above. The extracts were collected and then evaporated.

2.3 Separation of extracts by chromatographic methods

Thus, two types of chromatographic methods were used for fractionation and purification. These methods are: Thin Layer Chromatography (TLC), and Column Chromatography (LH-20 Seledex). The pure products obtained were identified by one-dimensional and two-dimensional nuclear magnetic resonance (NMR) spectroscopy (Nuzillard, 1998), (Le Guennec, 2015), (Guduff, 2018), (Menet, 2011). They are sent to Germany to Bielefield in the Department of Organic Chemistry.

2.4 Biological test

For the biological test, to facilitate application, the extract was prepared in two forms: cream of 5% and 10% raw snail extract and snail slime in the form of a solution. The mice were divided into 6 batches of 3 animals. Lot 1 animals were treated with slime. Lot 2 and 3 mice were treated with 5% and 10% extracts, respectively. Mice in Lot 4 were not treated while mice in the control (Lot 5) were treated with 100% Vaseline. Another batch (batch 6) was treated with OMF (Ody Fery Meva), a known healing product validated by IMRA. All these products were applied to the wound surface just after their creations. Then, once every 3 days for 20 days. Changes in the wound were noted and measured by direct planimetry: transparent plastic paper was placed on the wound and the wound contour was measured with a fine-tipped pencil (Bensegueni et al., 2007). Paper using a fine-tipped pencil. After each measurement, the wound was cleaned with absorbent cotton with 90° alcohol. Thus, the mean cure rates of the same lot were calculated and presented as mean average Tc ± s.e.m. The means were compared with each other using the student t-test. A $p < 0.05$ value was considered significant.

3 Results and Discussion

3.1 Results of maceration, extraction, fractionation and purification

The three extracts were obtained after maceration at room temperature. The ethanolic extract containing the polar products was the most abundant with a yield of 1.82%. Followed by the DCM/MeOH extract containing the moderately polar products with 1.07% yield and finally the hexanolic extract containing apolar products with a yield of 0.23%. Thus, the liquid-liquid partitioning by solvents of increasing polarity of 9.4 g of DCM/MeOH extract (50/50) allowed to obtain two fractions: DCM, ethyl acetate and aqueous fraction, which have a respective mass of 4.4 g; 1.4 g and the non determined aqueous extract.

3.2 Results on chemical screening

The three extracts were obtained after maceration at room temperature. The ethanol extract containing the polar products is the most abundant with a yield of 1.82%. Followed by the DCM/MeOH extract containing the moderately polar products with 1,Finally, hexanic extract containing apolar products with a yield of 0.23%. Thus, the liquid-liquid partition by solvents of increasing polarity of 9.4 g DCM/MeOH extract (50/50 made it possible to obtain two fractions: DCM fraction, ethylacetate and aqueous, which have a respective mass of 4.4 g; 1.4 g and the aqueous extract not determined. Student. A $p < 0.05$ was considered significant.

3.3 Results on the separation of extracts

Good separation of components was achieved using DCM/Hex eluent systems (8:2-v:v) and 100% DCM. The use of these eluent systems is necessary for chromatographic column fractionation and purification. On the other hand the separations are bad for hexanic and ethanolic extracts so the following work was done only on the DCM extract. Fractions with a nearby chromatographic profile should be grouped together to give a fraction by rating “Fx” but the X-index is in Roman digits.

Table 1 : Grouping of fractions

Pooled fraction	Name
F_1 à F_{34}	F_I
F_{35} à F_{49}	F_{II}
F_{50} à F_{73}	F_{III}
F_{74} à F_{90}	F_{IV}
F_{109} à F_{120}	F_V
F_{126} à F_{146}	F_{VI}

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For the F_I column, the appointment is F_{I-X} (with x ranging from 1 to 69). Fractionated by the eluent system DCM (3) / Hexane (7) of increasing polarity up to DCM (100%), the fractions were grouped into 11 fractions. Of the 11 pooled fractions, the F_{I-6} to F_{I-12} is called F_{I2}. LH20 sephadex column separation is required for F_{12} fraction purification. This fraction contains majority steroids on CCM. 45 fractions were obtained where they were grouped into 4 fractions. The thin-layer chromatographic profile of this extract. Fraction 16 is the majority and appears as white crystals noted F_{I2-2}. A rinse with ether was done and during this operation the soluble part in the ether undergoes a preparative thin layer chromatography to obtain a pure product of violet color on the CCM plate coded FR02 having 20 mg in mass. 71 F_{V-X} graded fractions (x ranges from 1 to 71) were obtained by fractionation of F_V., and collected into five other fractions (Diagram 5) graded F_{V-X} (x varies from I to V). Fractions F_{V-24}-F_{V-33} rated F_{V-III} have mono-tasks on CCM so they are pure. This column was made with very small amount of five drops per fraction.

3.4 Results on Spectral Analysis

Five spectra were recorded for the FR02 NMR analysis: Single-dimensional 1H spectrum; Single-dimensional ^{13}C spectrum Broadband decoupling (BB ^{13}C), Distortionless enhancement by Polarization Transfer (DEPT 135); two-dimensional proton-nuclear mononuclear correlation spectrumCOSY proton and two-dimensional proton-carbon heteronuclear spectra HMQC and HMBC. On the spectrum the peaks j, k, l, m, n in Figure 25 are very intense compared to the others of respective value corresponding to 3h (0.69; 0.88; 0.93; 1.02; 1.12). So these are $-CH_3$ methyls. The other protons are $-CH_2-$ and -CH-designated by peaks a, b, c, d, e, f, g, h, i with chemical shifts of 5.37; 3.53; 2.28; 2; 1.85; 1.53; 1.47; 1.35; 1.28 ppm.

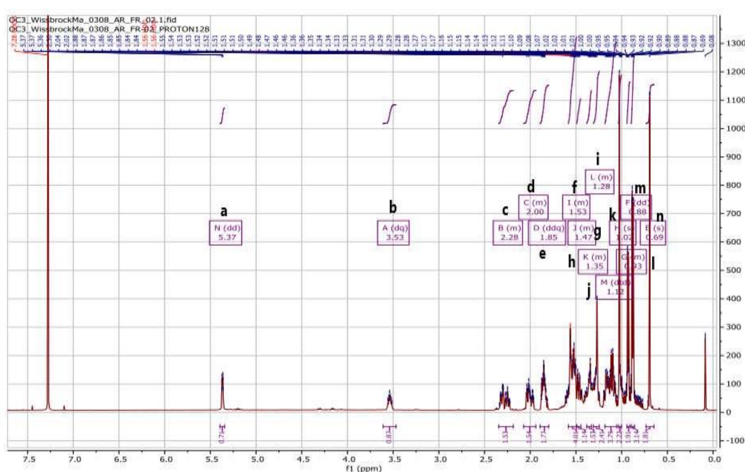
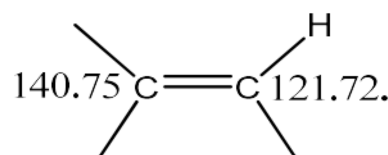


Fig. 1: Proton spectrum of FR02

The step height values of each proton is equivalent to the number of proton. In order to perform this proton number calculation from the bearing height; we consider the shorter 5.37 proton bearing height is equivalent to 1 proton. Based on this hypothesis, the others can be calculated by rule of three. Proton 5.37 ppm is an ethylene proton. A total of 45 protons were obtained for the total height of the bearing (27.66cm) corresponding to 0.71 cm for a proton.

The 13C Broadband carbon spectrum gives signals in the form of singulet with a value of between 140.75 the most debugged at the most shielded 11.87 ppm 26 peaks were seen on this spectrum but the triplet at 77.21 ppm is the peak of the solvent CDCl3. The appearance of proton spectrum and carbon 13, seen the carbon number, FR02 has the basic structure of a steroid. The chemical shift of carbon 71.81 to show the presence of a carbon containing hydroxyl group. The two carbon peaks at 140.75 ppm and 121.71 ppm are characteristic of quaternary and ethylene carbons belonging to a cycle. The proton spectrum gives information that there is only one ethylene proton which claims that 140.75 ppm may be a quaternary carbon; the DEPT 135 spectrum confirms that 121.71 ppm is a methine and the peak disappears at 140.75.



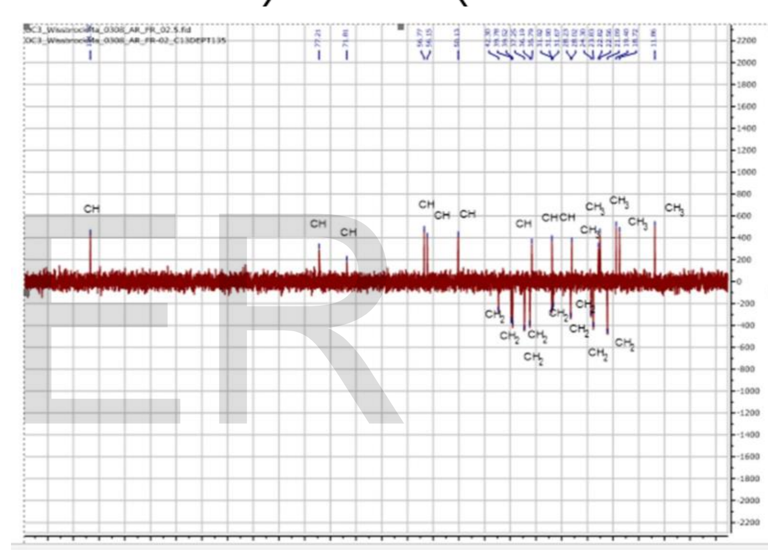


Fig. 2: Spectrum DEPT 135

DEPT 135 gives the number of carbon $-CH_2-$ and the -CH- and $-CH_3$. There are 11 carbons on the negative side of DEPT-135 so here there are 11 methylene groups $-CH_2-$ (Fig.2 , CH_2 area). The $-CH_3$ and -CH- are on the positive side and according to the count there are 8 -CH and 5 $-CH_3$ (Fig.2, CH and CH_3 area). So 24 carbons in total with the $-CH_2-$. The carbons with values 141.75 and 36.5 ppm do not appear on DEPT-135 so they are quaternary carbons. Carbon 31.92 is masked on Broadband but visible on the negative part of DEPT-135, it is a $-CH_2-$. These three spectra give us the following information:

- The number of carbon is 26 including 2 quaternary 141.75 and 36.51
- The basic structure is a sterol characterized by the existence of a hydroxide in position 3 of ring A and a double bond in C_5-C_6 in ring B which gives the sequence 1 of fig. 3.

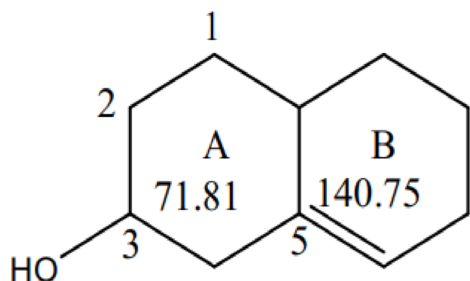


Fig. 3: Characteristic carbon sequence of beta-sitosterol

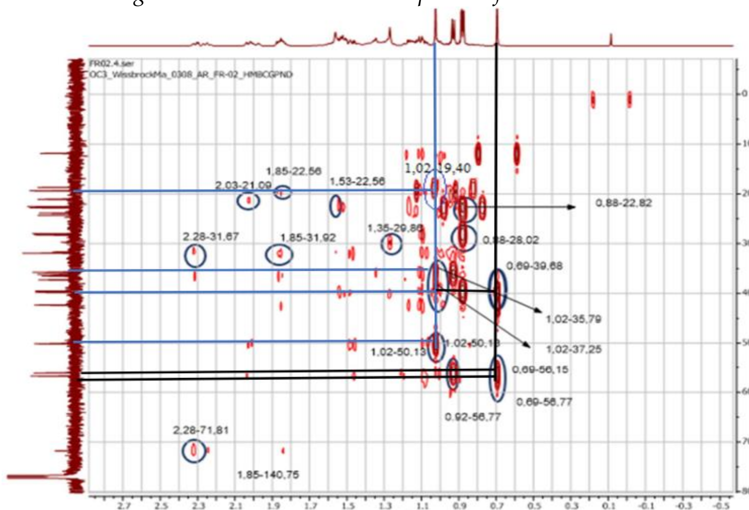


Fig. 4: Spectre HMBC

This spectrum gives us the correlations between proton-carbon distant from two and up to four bonds. The correlations between 80 ppm to 142 ppm and 5.37 ppm to 2.7 ppm are absent on the above spectrum but can be found in Appendix 3. The 5.37 proton correlates weakly to carbon 42.32 ppm, moderately correlated to carbon 36.51ppm and strongly correlated to carbon 31.92 ppm. Various correlations and some locations of carbons and protons were found on this spectrum. The location of the 121.72ppm carbon, the most deblind proton carrier "a" with a value of 5.37ppm, is used to determine the positions of the other correlations to the HMBC correlation intermediate. The proton "a" correlates directly with the carbon of value 121.72 ppm on the COSY spectrum. It correlates with carbon 140.75 ppm and 31.92 ppm and then correlates weakly with carbons 42.32 ppm and 36.51 ppm. The methyl proton 19.40 ppm also correlates with the carbons at 140.75; 37.79; 50.13 and 36.51 ppm.

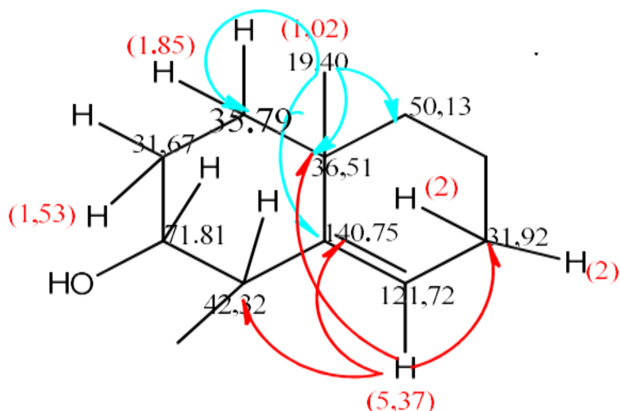


Fig. 5: Correlation seen in HMBC of methyl proton 19.40 and 5.37 with other carbons

The 0.69 ppm proton carried by methyl 11.86 ppm is correlated weakly with carbon 56.77 ppm but strongly correlated with carbon 56.15 ppm. It also has a strong correlation with carbon 42.32 ppm. But, this carbon already correlates with the proton 5.37 ppm which is at a very long distance from 11.86 ppm. By deducing, this carbon is another carbon lies near 11.86 views its correlation. The methyl 11.86 ppm also has a strong correlation with 39.78 ppm and 28.02. However, the proton of methyl 11.86 ppm correlates with carbon 23.83 ppm which is very far from 42.32. Therefore, the carbon peak at 11.86 ppm would be an overlapping signal. That is, two (2) carbons that resonate at the same frequency, so called "hidden carbon".

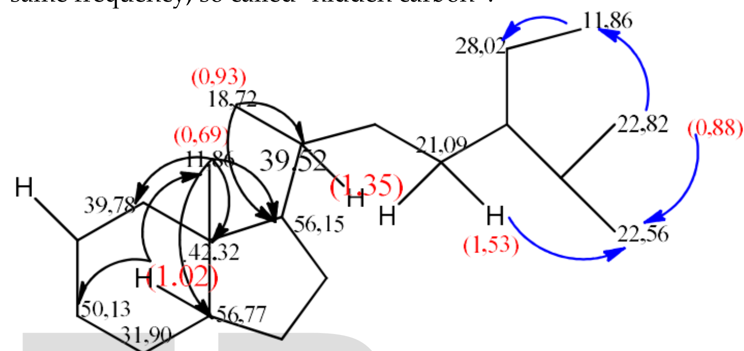


Fig. 6: Correlations of 11.86 and 18.72 methyl protons in HMBC

Correlations between proton-proton exist on this spectrum but some is very difficult to read. The proton "a" correlates with "d" while "b" correlates with c, e, f.

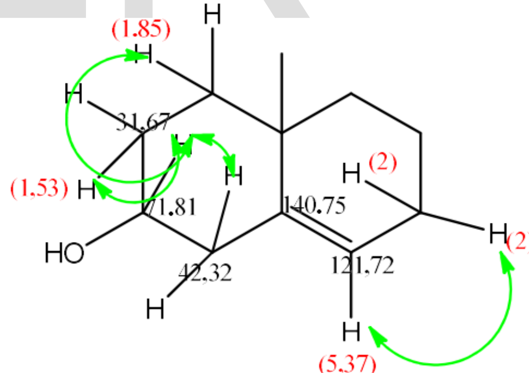


Fig. 7: COSY correlation

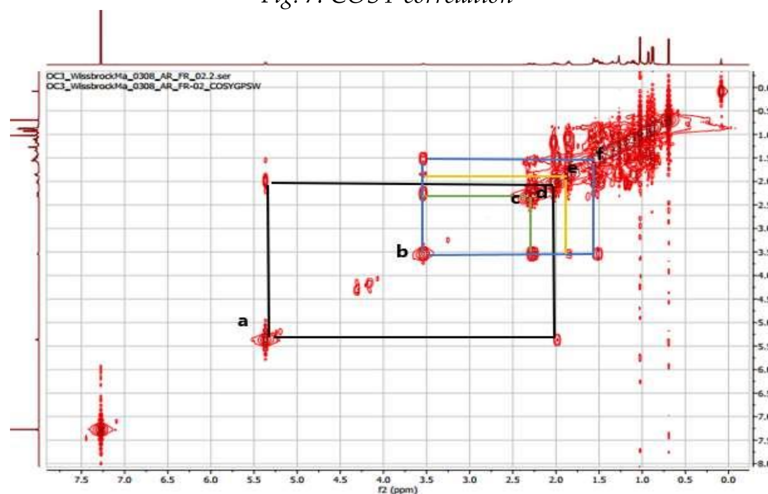


Fig. 8: Two-dimensional spectrum COSY

All this exploitable information above (COSY spectrum proton) on the 5 interpreted spectra can be used to determine the basic skeletons and some chemical displacements. However, some chemical shifts of carbons are missing like carbons number 11; 15, 16 and 22 and 25. To have complete information on the value of the chemical shifts of these carbons, we must make the comparison with those of the literature (Chaturvedula et Prakash, 2012 and Nyigo et al., 2016). The comparison of the chemical shifts of 13C FR02 with the values of the literature. The values of the unallocated carbons are 23.83; 28.23; 29.88; 35.79; 24.30; 39.78.

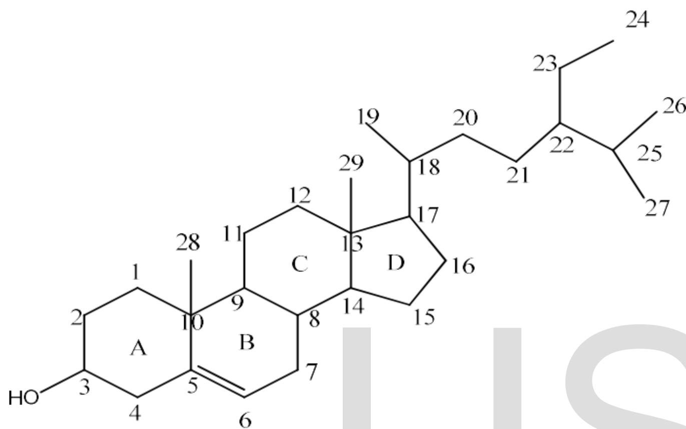


Fig. 9 : Numbering of the carbon skeleton of steroids according to the I.U.P.A.C. nomenclature

The summary of the information given by mono-dimensional NMR spectra (1H and 13C and DEPT 135), two-dimensional NMR spectra (COSY and HMQC and HMBC) and literature comparison values allows to determine the final sturcture.Hence the proposed structure is 17-(5-ethyl-6-methylheptan-2-yl)-10,13-dimethyl-1,2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydrocyclopentaphenanthren-3-ol for FR02.

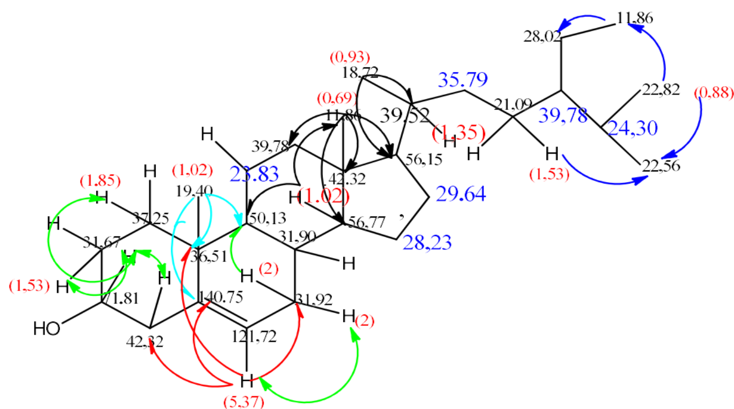


Fig. 10: Correlation COSY, HMQC and HMBC of FR02

The healing process takes 2 steps and lasts 30 days. The first step is tissue repair and lasts 15 days. The second stage leads to hair regrowth on the healed area. It is observed after 20 days of treatment. The mass of the paper corresponding to the wound surface of mouse 1 of batch 1 is 1.5 mg on the 10th day of the Test. This is equivalent to a wound surface of 0.375 cm² and a healing rate of 70% compared to the first day of measurement. For mice 2 and 3 in Lot 1, the respective rates on the same day were 31.915% and 51.064%. This gives an average healing rate of 50.993±7.343% for the slime-treated batch (batch 1).

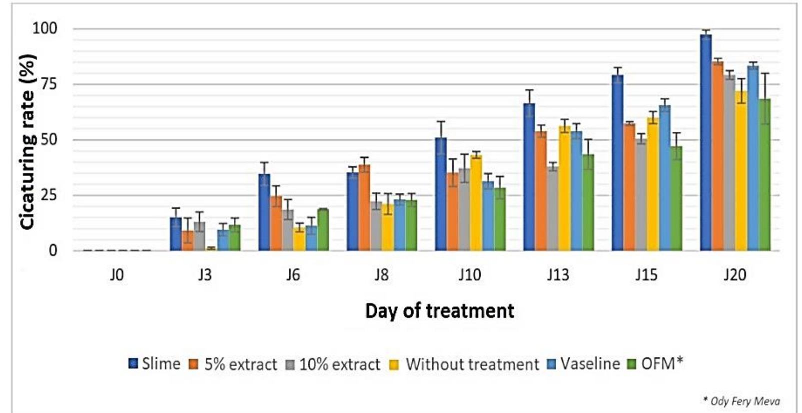


Fig. 11: Induced wound healing rate in mice as a function of treatment type and day

This histogram represents the average healing rates according to the type and day of treatment. These rates were calculated from the wound surface measured by direct planimetry every 3 days until wound closure. Based on this result, the rate of healing varies according to the time and the product tested. This rate increases from D0 to D20 and depends on the treatment received. The wound healing rate of the slime-treated wound was higher than that of the control (lot 4) during the 20 days of treatment. These differences were statistically significant at days 6, 15 and 20 ($p<0.05$). Wounds treated with slime (lot1) closed after 20 days (healing rate 97.334 ± 2.053%) whereas on the same day, the healing rate was 72.058 ± 5.573% for the wounds of the untreated mice (lot 4). These results suggest that for the mice treated with slime, the healing rate is significantly faster than that of the untreated control lot, regardless of the day of measurement. Two of the three mice in the slime-treated group showed complete healing and hair regrowth. This indicates that the slime has a healing effect. From day 6 to day 10, the healing rates of the wounds treated with the 5% and 10% cream were higher than those treated with the base cream. This difference was significant on day 8 for the batch treated with the 5% cream ($P<0.05$): a rate of 38.769 ± 3.309% vs. 22.309 ± 3.632% for the control lot (lot 4). On days 13 and 15, the respective healing rates were 37.949 ± 1.889 and 50.505 ± 2.333% in the animals treated with the 10% cream, compared with 53.927 ± 3.423% and 60.017 ± 2.724 in the control animals ($P<0.05$). The difference between the mean wound-healing rates of the cream-treated mice and those of the Vaseline-treated batch (corresponding negative control) was significant only at around days 8 to 15. From day 15 onward, healing and hair regrowth occurred similarly in all three batches (1, 2, and 5). The application of OFM also improved the wound healing rate on day 6 of treatment compared with the untreated

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batch ($P<0.05$). In view of these results, slime accelerates the wound healing process and seems to act on these two major phases. In contrast, the crude extract is only active during the initial and reparative phases. The healing action of these products could be due to the presence of alkaloids. Indeed, some alkaloids like allantoin have been widely known healing activities.

5 Conclusions

This work was done as part of the initiation to the research of the valorization of the biodiversity of Madagascar. We focused our research on animals of the genus *Achatina fulica*, to diversify the axis of research in our laboratory but also to justify the biological activity and to see from the chemotaxonomic point of view the metabolites present in this family. The chemical part of the work has shown that *Achatina fulica* presents several secondary metabolites such as alkaloids, steroids and lipids and free fatty acids. This justifies the literature on studies already done on this genus. It is the presence of alkaloid on this animal that confers its healing activity and its use in cosmetics. The medical benefits of this animal have been demonstrated.

Fractionation and isolation of the extract with dichloromethane yielded two steroids. Isolation and purification of the steroids were done by open silica column chromatography and preparative TLC. Identification of the isolated products was performed by NMR spectral and ESI-MS methods. Two types of steroids were isolated by chemical methods, one of which is systematically known as 17-(5-ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-ol.

As for the biology part, a biological study by direct planimetry method and statistical study were used to prove the healing effect of *A.fulica* extracts. The test of healing activity made on the slime and the raw extract gives us satisfactory results. For the slime, the two phases of the healing process were successfully tested (formation of the primary scar and maturation of the scar) with a healing rate of $97.334 \pm 2.053\%$ against $72.058 \pm 5.573\%$ for the control batch. On the other hand, the methanolic extract of 5% to 10% compared to vaseline did not have a good activity but only active during the initial and repair phases. In fact, it acted only during the last phase, but its activity increased when the percentage of the extract in the vaseline increased.

These two molecules were isolated for the first time from this species of snail (*A.fulica*). The chemical analysis made on this animal contributes to a great interest on the Chemotaxionomic level. Moreover, the present study has shown for the first time a healing effect of extract of *A.fulica* origin. The objectives set on this project have been achieved. And for the further work, it will consist in purifying and identifying other active compounds responsible for the healing activity. Some of them are supposed to be of alkaloid nature and others unknown that could have various interests in cosmetic and medical research.

6 Acknowledgements

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