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**Research Article**

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# New therapeutic precursors isolated from *Arbus precatorius (Fabaceae) a* plant used against infections in Benin

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**Abstract** We have contributed to the valorization of a flora plant of beninese used traditionally in antibacterial infections. We have prepared hydroethanolic extract from powdered dried leaves of *Arbrus precatorus (Fabaceae)*. We did a split bioguided from the crude hydro-ethanolic extract. We have isolated and purified by the series of methods chromatography two flavonoids from the active methanolic fraction of the crude hydro-ethanolic extract of the dried leaves of *A. precatorius*. The spectrometric analyzes carried out, namely mass spectrometry, proton and carbon nuclear magnetic resonance, made it possible to identify the following molecules. These are 5-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-4-oxo-4H-chromen-3-yl acetate and (E)-2-(4-methoxybenzylidene)-5,7-dihydroxy-2,3-dihydroinden-1-one. The added value of the work lies in the fact that these two molecules have never been identified before in this plant and therefore constitute an avenue for developing a therapeutic arsenal to fight recurrent microbial infections.

**Keywords** isolation, chromatographic methods, spectrometric analyzes

## 1. Introduction

*Abrus precatorius* is a shrub with creeping, hairy roots. The vine stem climbs up to fifty meters in height and its vine wraps around the trees located near it. The alternate, beautiful light green leaves measure between 5 and 13 cm long and are made up of 7 to 10 pairs of obovate-shaped leaflets. The rosary-shaped inflorescence appears as small pink to purple spikes. In traditional medicine, *Abrus precatorius* is recognized as very effective for the treatment of colic in newborns. The seed extract of this plant was used in pharmacology to treat ophthalmic problems. A decoction of

red pea leaves helps treat diabetes. Leaf extracts diluted in palm wine give a galactogenic composition. In India, these leaves are used to cure fever, stomach disorders, asthma and bronchitis (Suralkar and Kasture, 2013). The sweet-tasting leaves are very popular in Madagascar and Senegal in the treatment of coughs, especially childhood coughs. *Abrus precatorius* is classified among the antitussive plants (Gairola et *al*., 2010). In Nigeria, leaf decoction is used in the treatment of diabetes (Ezuruike and Prieto, 2014). In Ivory Coast, an ethnobotanical survey showed that the leaves of *A. precatorius* are used in the south of the country, among the Abbey and Krobou people of Agboville, in a drink to facilitate childbirth for women (N'guéssan et *al*., 2010). The aqueous extract of the leaf and stem is also taken orally as an aphrodisiac by men and to facilitate childbirth in women. Also in West Africa, the dried roots are used to prepare a decoction administered orally to treat bilharzia, gonorrhea, chest pains and as an aphrodisiac, an antiemetic and antiparasitic against tapeworm. The powder from the grains is used by several African tribes as an oral contraceptive (Garaniya and Bapodra, 2014). The aqueous extract of the grains is used for the treatment of epithelial tissue cancers. The whole plant is used for the treatment of venereal diseases, headaches and snake bites (Iwu, 2014).

These different uses show the importance of this plant in traditional medicine. In this study, we wanted to characterize the methanolic fraction active on certain strains of bacteria of the hydro-ethanolic extract of the dried leaves of this plant.

## 2. Materials and Methods

### 2.1 Plant material

The plant material consists of dried leaves of *Arbus precatorus* collected in December 2020 at Abomey-Calavi and identified in the National Herbarium of the University of Abomey Calavi. The leaves of the harvested plant were washed then dried at room temperature in a ventilated room of the Pharmacognosy laboratory for three weeks before being reduced to powder using an electric plant grinder.

### 2.2 Preparation of the raw extract

The extraction was made from the dried leaves of *Arbus precatorius* by mixing 50g of powder in 500 mL of a hydro-ethanolic mixture (40V/60V respectively) for 48 hours. After respective filtration on Whatman No. 1 paper, the filtrates obtained were evaporated using a rotary evaporator at 40°C. The residues of this filtrate were dried in an oven for 48 hours at 40°C to obtain the dry extracts (Hougbeme et *al*., 2014)

### 2.3 Liquid-liquid fractionation

Liquid–liquid extraction is carried out by intimate contact of the solvent with the solution in a separatory funnel. The solution to be fractionated consists of the crude hydro-ethanolic extract dissolved in 50 mL of distilled water. We successively carried out the extraction with 500 mL of the following solvents: cyclohexane, dichloromethane, ethyl acetate and methanol. The different fractions (phase resulting from the operation containing the extracted solutes) collected were evaporated in a rotavapor at 40°C.

### 2.4 Antibacterial test

For the antibacterial test, we used four reference strains provided by the Applied Microbiology and Pharmacology of Natural Substances Research Unit (table 1).

**Table 1:** Strains used for biological study

| Strains | Reference | Strains | Reference |
|---|---|---|---|
| *Pseudomonas aeroginosa* | ATCC 27853 | *Staphylococcus aureus* | ATCC 25923 |
| *Salmonella typhi* | ATCC 14028 | *Escherichia coli* | ATCC 25922 |

We proceeded to determine the Minimum Inhibitory Concentration (MIC) in liquid medium and the Minimum Bactericidal Concentration (CMB) in solid medium of the different fractions.

The (MIC) is the lowest concentration of the substance for which there is no growth visible to the naked eye after an incubation time of 18 to 24 hours while the (MBC) is the lowest concentration of the substance which leaves at most 0.01% of germs surviving.

For the determination of the MIC using a platinum loop, a certain quantity of bacterial strain previously preserved in the agar of Mueller Hinton was removed by scraping then subcultured by quadrant on a plate of ordinary agar then incubated at 37°C for 18 to 24 hours in order to have isolated colonies. After this incubation time, 3 to 5 colonies were taken, then inoculated in 10 mL of broth then incubated at 37°C for 3 to 5 hours. Meanwhile, the concentration ranges of each plant extract were prepared according to the double dilution method in liquid medium with a geometric progression of the concentrations of the extracts. They generally vary from 0.781 to 100 mg/mL. For each concentration range, 0.2 mL was taken and then placed in a specific tube from a series of experimental tubes. In this series called test series, one tube served as a growth control (containing 0.2 mL of sterile distilled water). After 3 to 5 hours of incubation, 0.2 mL of the inoculated broth was taken, then homogenized using a "VLEP Scientifica" type vortex shaker in 20 mL of sterile Mueller Hinton broth. Then, 1.8 mL of this last broth was taken to complete the volume (0.2 mL) of the tubes of the test series to 2 mL. Alongside the test series, a reference series was prepared. In the latter, the experimental tubes each contained 0.2 mL of each concentration of plant extract previously prepared and the control tube 0.2 mL of sterile distilled water. To all the tubes of the reference series, 1.8 mL of sterile broth was added. All the experimental tubes of the test series and the experimental tubes of the reference series were homogenized using a "VLEP Scientifica" type vortex shaker then incubated at 37°C for 18 to 24 hours (Nassif et *al*., 1990 ; Okou et *al*., 2015). One day after incubation, the minimum inhibitory concentration (MIC) was determined by direct reading, with the naked eye, in daylight. To determine this parameter, we compared concentration by concentration, the tubes of the test series with those of the reference series in search of the absence of turbidity (Marmonier, 1990; Okou, 2012). This determination of the MIC was repeated during three successive experimental tests.

For the determination of the CMB, the growth control control tube of a given bacterial strain was diluted 10 in 10 up to $10^{-4}$ according to a geometric progression of ratio $10^{-1}$. Then the various dilutions were inoculated on a Mueller Hinton agar plate, on 5 cm streaks using a calibrated loop (Box A). To better appreciate the evolution of the sensitivity of the bacterial strains used in the presence or absence of plant extract, inocula obtained from a given bacterial strain were inoculated on a Mueller-Hinton agar plate on streaks of 5 cm using a calibrated loop. The inocula seeded were the inoculum from the control growth control tube, the inocula where turbidity was not visible and some inocula preceding the tube which made it possible to determine the MIC (high bacterial load) (Box B). Finally, Boxes A and B were incubated at 37°C for 18 to 24 hours. After this incubation time, comparison of the number of colonies on the streak, at the $10^{-4}$ dilution of Box A with that of each streak of Box B, made it possible to determine the minimum bactericidal concentration. According to Marmonier in 1990:

- -the CMB/MIC ratio ≤ 4, the substance tested is bactericidal.
- -the CMB/MIC ratio > 4, the substance tested is bacteriostatic.

**2.5 Purification and Isolation**

We fractionated the active methanolic extract obtained by bioguided fractionation and its sub-fractions using the liquid-solid chromatography technique on a column at atmospheric pressure (CPA). The stationary phases that we used are successively RP 18 silica gel (40-63 μm); normal silica gel (60 PF 254) and Sephadex LH 20 gel; These different gels were dissolved in methanol (30 g in 150 mL of methanol) then poured into a glass column. The eluent is under atmospheric pressure, enters at one end and exits at the other. It can be a single solvent for conditioning or a mixture of solvent for different gradients. The chromatographic conditions of the partition for the ethyl acetate fraction and these sub-fractions are presented below:

Dichloromethane: 100% (column conditioning)
Dichloromethane – Ethyl acetate: 90 – 10
Dichloromethane – Ethyl acetate: 70-30
Ethyl acetate - MeOH: 50 – 50
Ethyl acetate - MeOH: 20 – 80
MeOH 100%

Elution: 200 mL solvent gradients

Collection flow: 5mL/tube at 1 drop/second
Deposit: 200 mg of solubilized fraction in 10 mL MeOH
The analysis of the subfractions obtained was carried out by thin layer chromatography with the solvents Ethyl acetate/ MeOH /$H_2O$(v/v/v) 81-11-8 as mobile phase and alcoholic potassium hydroxide as developer. 10% which highlights the presence of phenol acids, flavonoids and quinones (Bruneton 1999). The subfractions pooled after analytical TLC were re-submitted to subsequent fractionations on CPA. The fractions at the end of purification are passed through an SPE column with the Sephadex gel for exclusion chromatography which separates the compounds according to their size and molecular weight (Houngbeme et al, 2015). Figures 1 and 2 below describe the stages of two major sub-fractions collected and from which we were able to isolate the molecules.

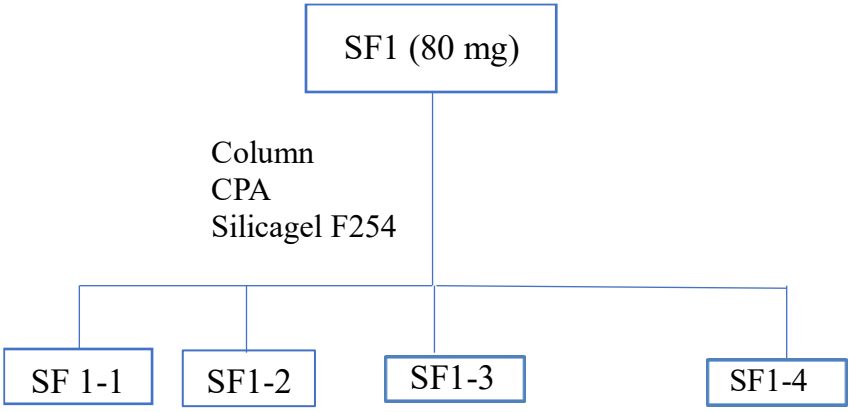



*Figure 1: Diagrams showing the steps in the purification of the SF1 subfraction*

The selected S/F1 fraction was purified again on F 254 silica gel and gave 4 SF-1 subfractions; SF-2; SF-3; SF-4. The SF-1 subfraction reveals two well-separated spots at UV 254 nm. So we applied preparative TLC to scratch the majority spots. The majority scratched spots are then separated by exclusion chromatography (figure 2).

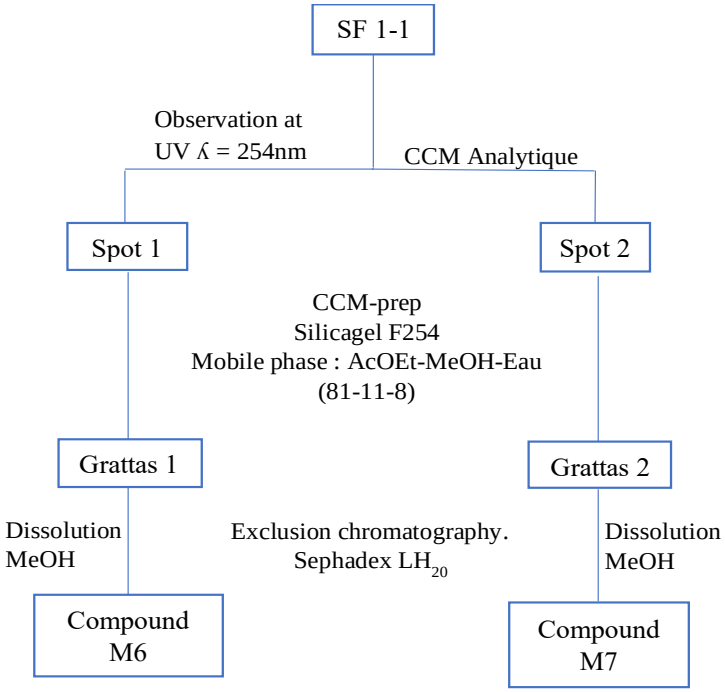



*Figure 2: Diagrams showing the stages of purification of the SF1 subfraction*

## 3. Results and Discussion

### 3.1 Extraction yield

Extracts yields obtained were summarized in the table 2

**Table 2** Extraction yield

| Plant material | Extract | Mass | Yield |
|---|---|---|---|
| Raw extract (20g) | Extract $C_6H_{12}$ | 0.25g | 1.25% |
| | $CH_2C1_2$ extract | 0.29g | 1.45% |
| | AcOEt extract | 3.58g | 17.9% |
| | MeOH extract | 3.4g | 17% |
| | Aqueous Extract | 9.25g | 46.2% |

### 3.2 Collection of sub-fractions

The different sub-fractions are summarized in the following table.

**Table 3:** Distribution of tubes collected after TLC and Revelation

| Subfractions | Tubes bundled | Mass obtained (mg) |
|---|---|---|
| S/F1 | T 1-13 | 80 |
| S/F2 | T 14-20 | 50 |
| S/F3 | T 21-40 | 20 |
| S/F4 | T 41-60 | 14 |
| **Total / Yield** | | **119/59.5%** |

### 3.3 Antibacterial test of the fractions obtained

Among fractions, the methanolic fraction was more active than the other (table 4).

**Table 4:** Table of values obtained for the CMB and the MIC

| Types of fractions | CMI | CMB |
|---|---|---|
| Cyclohexane fraction | 0.31 - 5 mg/mL | 1.25 - 10 mg/mL |
| Dichloromethane fraction | 0.31 - 5 mg/mL | 1.25 - 20 mg/mL |
| Ethyl acetate fraction | 0.31 - 5 mg/mL | 1.25 - 10 mg/mL |
| Methanolic fraction | 0.15 - 2.5mg/mL | 0.62 - 20 mg/mL |

### 3.4 Structure of isolated molecules

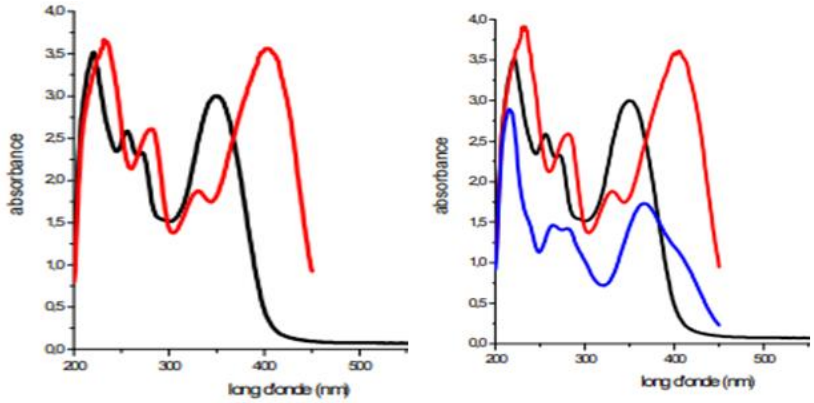
The processing of information from different spectra made it possible to elucidate the structure of the isolated compounds.

### 4. Spectrometric data of compound $M_6$

- **ESI-MS (m/z)** : 372.011 g/mol ($[M-H]^+$) ; 354.011 ([M]-17) ; 340.013 ([M]-31) ; 312.09 ([M]-59).
- **$^{13}$C NMR ($CD_3OD$, 100 MHz, δ in ppm )** : 178.1 (-CO-, cyclic); 168.3 (-CO-, ester); 168.9, 133.8 (C=C-ethylenic); 168.4, 162.98, 159.5, 152.1, 145.3, 125.03, 120.14, 117.32, 112.17, 105.7, 97.12, 96.54 (C-aromatic); δ 55.7, 56.1 (O-$CH_3$), 21.13 ($CH_3$).
- **$^1$H NMR ($CD_3OD$, 400 MHz, δ in ppm )** : 8.5 (singlet, 1H, -OH-aromatic); 6.73-5.56 (massif, 5H, H-aromatics); 3.75 (singlet, 3H, -$OCH_3$); 3.69 (singlet, 3H, -$OCH_3$); 1.98 (singlet, 3H, $CH_3$).

Compound **$M_6$** shows fluorescence under wood light at 365nm. It reacts with NEU reagent with yellow fluorescence. The **$M_6$** molecule therefore belongs to the family of flavonoids, that is to say a flavone or a flavonol substituted at $C_3$.

In order to identify the different substituents on the flavonoid rings , we carried out some standard chemical tests. Thus, the UV absorption of the mixture of compound M $_6$ with sodium hydroxide is greater compared to the methanolic solution of M6. Which means that M6 contains free hydroxide groups in this case the 4' position. Likewise, the product of the reaction between M6 and the aluminum chloride $AlCl_3$ in a hydrochloric medium has a lower optical density than the methanol control. Which means that the -OH hydroxyl group at position 5 is free.




- Pic in black: methanol solution
- Red peak: solution with NaOH
- Blue peak: solution with $AlCl_3$ in HCl medium

*Figure 3: UV absorption spectra of $M_6$*

Furthermore, the addition of sodium acetate caused a decrease in optical density compared to the Methanol control. We can therefore conclude that this flavonoid has at position 7 a blocked hydroxyl group, that is to say O-methylated.

Taking into account the information provided by UV spectrometry, we can attribute to compound M6 the provisional profile in the figure 4 below:

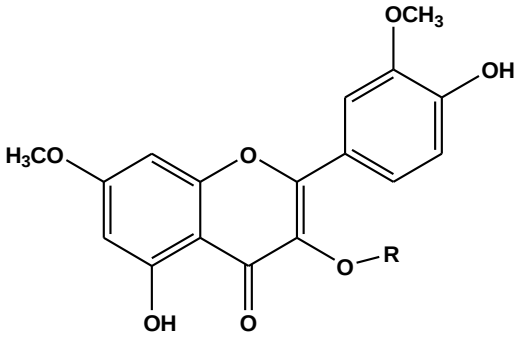



*Figure 4: Structural profile of compound **$M_6$***

The mass of the molecular ion obtained by positive impact mode $[M-H]^+$ gave 372.011 g/mol, which corresponds to molecular molar mass M = 371.011 g/mol, which approaches that of the proposed profile when R is the acetyl group:-$COCH_3$. The appearance of the fragment at [M]-59 responds to the loss of an O-acetyl group and therefore R is an acetyl group. Other main peaks including ([M]-OH); ([M]-$OCH_3$) indicate that the probable structure of **$M_6$** is that of figure 5 below :

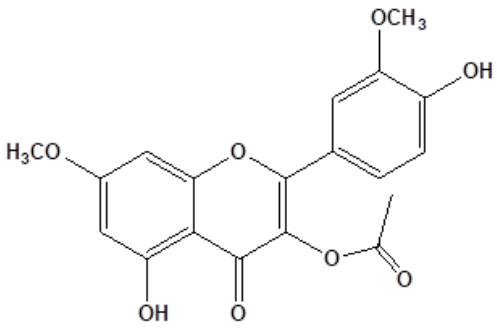



*Figure 5: Probable structure of compound M 6*

The chemical shifts of protons and carbons shown respectively by the proton NMR and carbon-13 NMR spectra clearly show the different constituent atoms of the proposed molecule after mass spectrometry. Thus, the aromatic, O-methyl, acetyl protons appeared as well as the carbons of the carbonyl group, ester, and aromatic then methyl carbons. These data confirm the structure proposed above. Compound **M6** (***5-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-4-oxo-4H-chromen-3-yl acetate***) is a substituted derivative of padmatine. This is 3-O-acetyl-3'-methoxypadmatine, which has never been previously isolated from this plant.

**5. Spectrometric data of compound: M 7**

- **MS (m/z )** : 286.03 g/mol ($[M-H]^+$ ) ; 285.03 ($[M]^+$) ; 269.03 ($[M-H]^+$ -17) ; 255.03 ($[M-H]^+$ -31); 179.03 ($[M-H]^+$ -107).
- **$^{13}$C NMR ($CD_3OD$, 100 MHz, δ in ppm )** : 192.2 (-C=O); 165.98-159.7 (O-substituted aromatic-CH); 127.2-114.4 (substituted aromatic-C-OH); 139.2 & 132.8 (aliphatic-CH ethylene) ; 55.8 (-$OCH_3$); 35.2 (-$CH_2$-).
- **$^1$H NMR ($CD_3OD$, 400 MHz, δ in ppm )** : 8.6 (s, 1H, OH-aromatic); 7.96 (s, 1H, OH-aromatic); 7.56 (s, H-C=C); 7.20-6.18 (m, 6H, H-aromatic); 3.72 (s, H-methyl); 3.21 (s, 2H, -$CH_2$-).

UV absorption at 365 nm of compound **$M_7$** shows that this compound belongs to the class of flavonoids or their precursor chalcone or even aurone. The $^{13}$C NMR spectrum shows a strongly deshielded peak at δ 192.2 ppm. Which corresponds to the presence of a carbonyl group in the molecular chain. The spectrum also shows peaks at δ 127.2-114.4 ppm which correspond to phenolic carbons (aromatic-C-OH). The presence of an aliphatic ethylene group at δ 139.2-132.8 allows us to reject the hypothesis that the compound is a flavonoid, but rather would be a substituted aurone. The presence of peaks at δ 55.8 (-$OCH_3$) and δ 35.2 (-$CH_2$-) indicates that the aurone compound is substituted by a methoxy group.

The $^1$H NMR spectrum shows aromatic protons at δ 7.20-6.18 ppm (Houngbèmè et *al*., 2015; Tokoudagba et *al*., 2022); two phenolic protons which appear as a singlet with a chemical shift δ 5.0 ppm. The appearance of a signal at δ 7.56 ppm indicates the presence of an aliphatic ethylene proton in the molecular skeleton of the compound (HC=C). In addition, the singlet at δ 3.72 (s, H-methyl) indicates the existence in the substituents of the methoxy group. In view of the proton and $^{13}$C NMR spectrometric data, we believe that compound **M7** has the probable structure presented in figure 6.

The molecular weight in positive mode $[M-H]^+$ gave 286.03 g/mol, which corresponds to the molecular molar mass M = 285.03 g/mol. This value corresponds to the calculated mass (M = 285 g/mol) for the provisional structure shown in Figure 6 above. The different characteristic fragments obtained are: 285.03 ($[M-H]^+$-H); 269.03 ($[M-H]^+$-OH); 255.03 ($[M-H]^+$-$OCH_3$). These data confirm those obtained by NMR.

Comparison of theoretical and experimental spectra shows that the values of the chemical shifts of protons and carbons are close with a non-significant difference which could be due to the nature of the solvent used and the frequency applied during the recording of the different spectra. Taking into account the analyzes of the available spectral data, the provisional structure of compound $M_7$ is confirmed in figure 6 below:

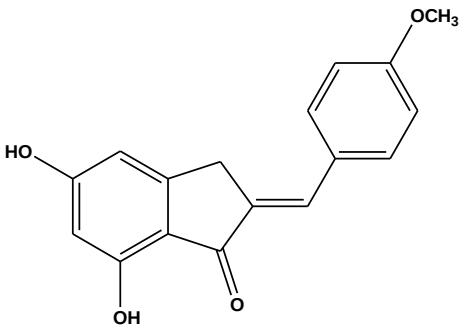



*Figure 6: Structure of compound M7 : (E)-2-(4-methoxybenzylidene)-5,7-dihydroxy-2,3-dihydroinden-1-one*

## 5. Conclusion

We carried out a bioguided fractionation of the crude hydro-ethanolic extract of the dried leaves of *Arbus precatorius* (Fabaceae) which allowed us to obtain an active methanolic fraction. The purification of this fraction allowed us to isolate two molecules which are two flavonoids. This fraction rich in flavonoids can be an alternative to the development of a therapeutic arsenal to fight against cancer and cardiovascular diseases.

## Compliance with ethical standards

**Conflict of Interest**: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Ethical Approval:** This article does not contain any studies with human participants or animals performed by any of the authors.

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