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Effect of methanolic and ethyl acetate extracts of the rhizomes of *Curcuma mangga* acclimated to Congo on the production of reactive oxygen species (ROS)

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Abstract
The effect of extracts from acclimated *C. mangga* rhizomes from Congo on the production of reactive oxygen species (ROS) was evaluated on leukocyte cells obtained by hemolysis of human blood. Two types of extracts were tested, the methanolic extract and the ethyl acetate fraction. The results show that both extracts significantly reduce the production of ROS. However, the extract obtained with ethyl acetate is more active with an inhibition percentage of 63%, compared to the methanolic extract which inhibits it by up to 55%. This significant activity of the ethyl acetate extract would be linked to the major compounds identified in this extract, namely Zerumin A and (E)-labda-8(17), 12-dien-15, 16- dial.

Keywords: *Curcuma mangga*, reactive oxygen species (ROS), Zerumine A et le (E) -labda-8(17),12-dien-15,16-dial

1. Introduction
Reactive oxygen species (ROS) are a type of free radicals produced by the body. During oxidative stress, their concentration increases and has negative consequences on DNA, lipids, and proteins. This homeostatic imbalance is at the origin of numerous pathologies such as inflammation, atherosclerosis, cancer, diabetes, and neurodegenerative diseases. Plant extracts rich in bioactive molecules can restore the balance between the production and consumption of ROS and thus prevent these diseases. *Curcuma mangga* (Zingiberaceae) belongs to the genus *Curcuma* which includes several species used as spices or as therapeutic agents in traditional medicine in tropical countries (Bikindou *et al*., 2020) [6]. Some species of this genus are used to treat bronchial conditions, dysentery, and bacterial and viral infections (Akarchariya *et al*., 2017; Chuakul and Boonpleng, 2003; Basaka *et al*., 2010) [1, 10, 4]. Previous studies on rhizome extracts showed anti-inflammatory and anti-cancer effects on several cell lines such as MRC-5, MCF-7, HCT116 and HT-29. These extracts also showed antidiabetic, hepatoprotective, antirheumatic, hypotensive, antioxidant, antimicrobial and hypocholesterolemic effects (Sikha *et al*., 2015; Afzal *et al*., 2013; Krup *et al*., 2013; Chen *et al*., 2008; Reanmongkol *et al*., 2006; Wilson *et al*., 2005; Angel *et al*., 2014; Mau *et al*., 2003) [18, 2, 11, 9, 16, 19, 3, 13]. *Curcuma mangga* is a species native to the island of Java which was introduced into several regions of Congo and has become subspontaneous. People use the leaves to wrap fish when preparing it for stewing, the leaves thus provide a spicy flavor. The essential oils of the leaves and rhizomes of this species have been the subject of publications (Bikindou *et al*., 2020) [6]. The preliminary study of non-volatile extracts from the rhizomes obtained with several solvents showed that the methanolic extract was the most active (Bitemou *et al*., 2020) [6]. The objective of this work is to evaluate the effect of methanolic and ethyl acetate extracts of rhizomes on the production of reactive oxygen species (ROS).

2. Materials and Methods
2.1 Plant Material: The rhizomes were collected in Loukoko (Pool Department). The collected plant material was authenticated in the herbarium of the National Institute for research in Exact and Natural Sciences (IRSEN).

A specimen was deposited under the number 1423. The plant material was then dried in the shade, away from light for two weeks then reduced to powder using a biomix type crusher.

2.2. Cell Material

The effect of the extracts on ROS production was evaluated on leukocytes and monocytes in whole blood, collected from healthy volunteers (n=3). These cells were obtained by hemolysis of blood by the action of a mixture of 1.658 g of ammonium chloride (NH_4CL, sigma aldrich), 0.2 g of sodium bicarbonate ($NaHCO_3$, sigma aldrich) and 0.0074 g of ethylene diaminetetraacetic acid (EDTA, sigma aldrich) prepared in 200 mL of sterile water.

2.3 Extraction and isolation of compounds

50 g of the rhizomes powder were macerated in 300 mL of methanol, the mixture was left stirring for 72 h. The mixture was then filtered. The extractions were repeated three times. The filtrates thus obtained were combined and then evaporated to dryness. The dry extract obtained was partitioned into solvents with increasing polarity such as cyclohexane (Sigma aldrich, 200 mL x 2), ethyl acetate (sigma aldrich, 200 mL x 2) and water. Three fractions were obtained, namely the cyclohexane fraction, the ethyl acetate fraction, and the water fraction. The ethyl acetate extract was fractionated on a conventional column containing 100 grams of silica gel with the ethyl acetate/cyclohexane mixture as eluent. Follow-up by thin layer chromatography was carried out, similar fractions were mixed. In total, 8 fractions were obtained (F1-F8), fraction F6, presenting fewer compounds after TLC, was fractionated. Five subfractions were obtained (F6.1-F6.5). The F6.3 subfraction was analyzed by liquid chromatography coupled with mass spectrometry (Figure 1).

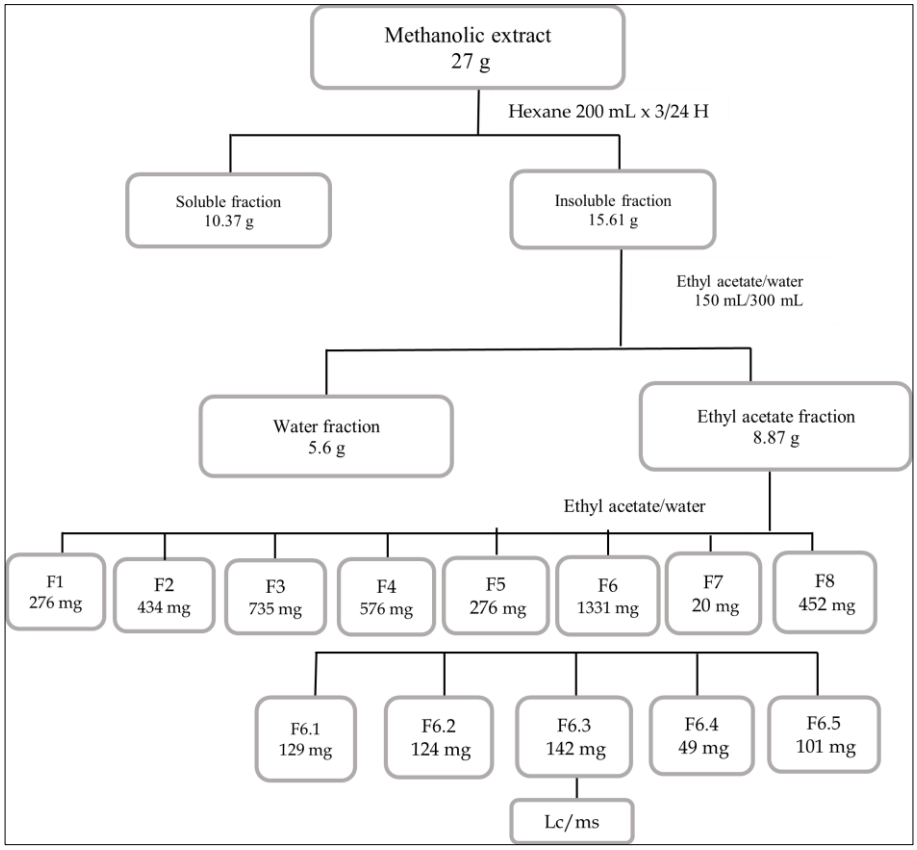

Fig 1: Compound isolation and identification

2.4 LC-MS analysis

The F6.3 fraction was analyzed on a UHPLC Ultimate 3000 RSLC and an Orbitrap Q-Exactive (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with a DAD detector and an Uptisphere Strategy C18 column (250 4.6 mm, 5 m, Interchim, Montluçon, France). The operating conditions are as follows: spray voltage 3 kV; capillary temperature 320 °C; auxiliary gas temperature 400 °C; flow rate of sheath 50, scavenging 10 and auxiliary gas (nitrogen) 2 arbitrary units; voltage of the collision cells between 10 and 50 eV. The analysis data were obtained at a resolution of 70,000. X Calibur software (Thermo Fisher Scientific Inc., City, MA, USA) was used to process the raw data. Compound identification was obtained in negative mode. The mobile phase consisted of the mixture of formic acid (0.1% v/v) in water (phase A) and formic acid (0.1% v/v) in acetonitrile (phase B). The elution gradient of phase A was 100% (0 min), 80% (10 min), 73% (35 min), 0% (40 to 50 min), and 100% (51 to 60 min). The flow rate was 0.8 mL/minute and the injection volume was 5 µL.

2.5 Effect of extracts on cell viability

The cells were incubated with the extracts at different concentrations (10, 25, 50 and 100 µg/mL) on a 96-well plate in the presence of resazurin (25 µg/mL, sigma aldrich), for 2 hours under an atmosphere of CO_2 (5%) and at a temperature of 37 °C. The cells were stimulated or not with phorbol 12-myristate 13-acetate (PMA 1 M, Sigma Aldrich). The fluorescence was read a 525 nm using a Fluoroskan Ascent FL® (Thermo Fisher Scientific, Illkirch, France). The percentage of cell viability was calculated relative to a control normalized to 100%.

2.6 Effect of extracts on ROS production: The effect of methanolic crude extract and ethyl acetate fraction on reactive oxygen species (ROS) production was evaluated on a 96-well plate. Indeed, 200 µL of cell suspension were incubated with 20 µL of extract at different concentrations (10, 25, 50 and 100 µg/mL), 10 µL of dihydrorhodamine 123 (DHR 10 mM, Cayman Chemical Company), 10 µL dimethyl sulfoxide (DMSO 100%, sigma aldrich). The cells were stimulated or not with 10 µL of phorbol 12-myristate 13-acetate (PMA 1 mM, Sigma Aldrich). Fluorescence was read every five minutes for two hours at 525 nm using a Fluoroskan Ascent FL® (Thermo Fisher Scientific, Illkirch, France). The experiments were carried out in triplicate., and the percentage of ROS production was calculated relative to a control normalized to 100%.

3. Results and Discussions

3.1 Isolation and determination of compound structures

Analysis of the chromatogram of fraction F6.3 (Figure 2) shows four major peaks, namely peak 7 at the retention time (Tr) equal to 1.85 min, peak 8 (Tr=1.87), peak 9 (Tr=1.99) and peak 10 (Tr=2.04). The identification of peaks 9 and 10 was made by comparison of their mass spectra with those of the databases.

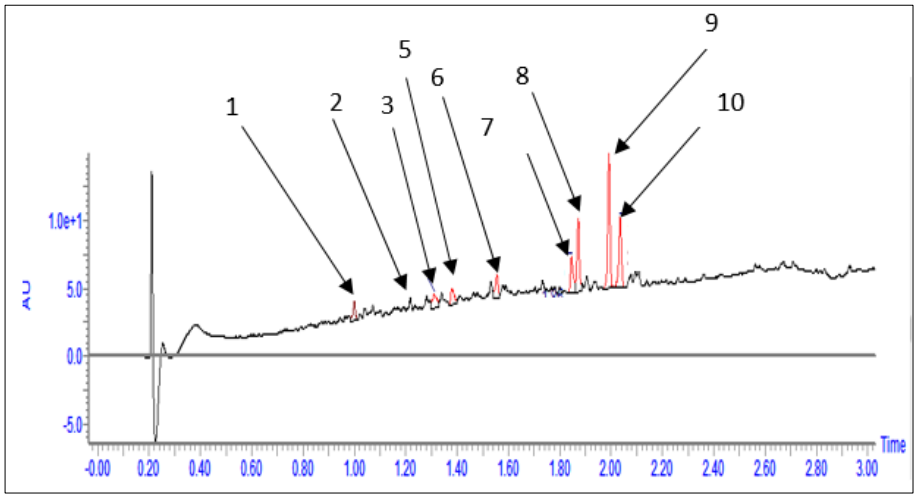

Fig 2: Chromatogram of fraction F6.3 of the ethyl acetate extract of *Curcuma mangga* rhizomes

Peak 9 whose molecular ion has a molar mass MH+ =305.2 with a characteristic dimer of molar mass M=611.5 g/molL seems to correspond to the compound (E) -labda-8(17), 12-dien-15, 16-dial (Figure 5).

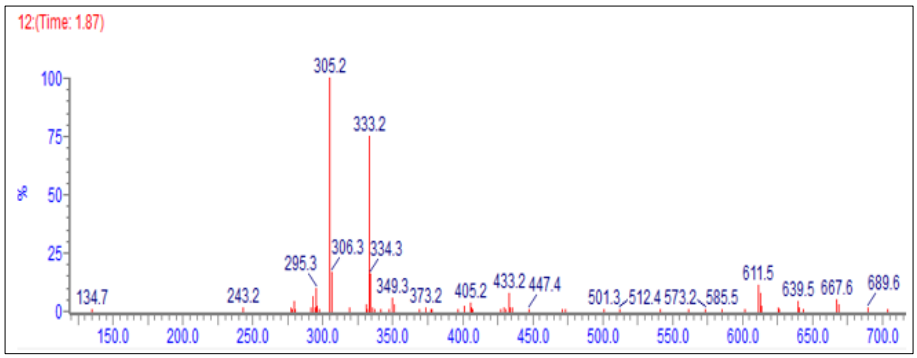

Fig 3: Mass spectrum of peak 9

Likewise, Peak 10, whose molecular ion has a mass MH+ = 317.2 with a characteristic dimer of molar mass 635.5 g/mol (Figure 4), seems to correspond to Zerumin A (Figure 5).

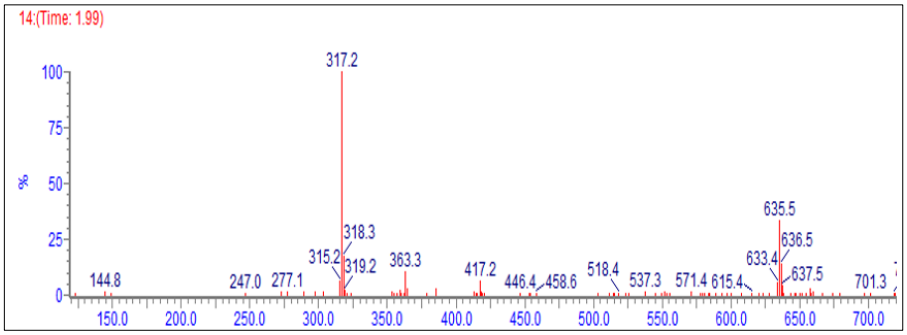

Fig 4: Mass spectrum of peak 10

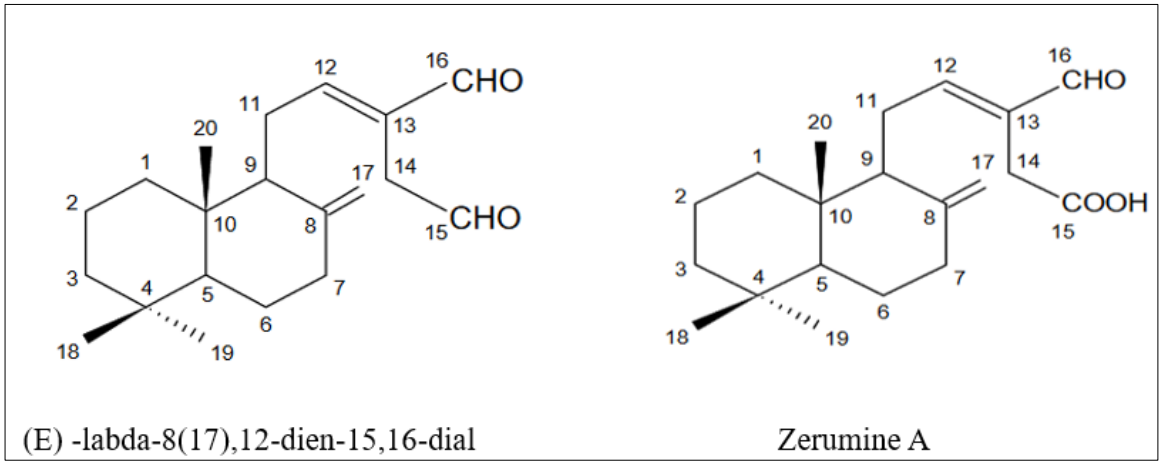

Fig 5: Chemical structure of identified compounds

The two compounds (figure 5) identified in the rhizomes of *C. mangga* acclimated to Congo have already been identified in the rhizomes of *C. mangga* acclimated to Indonesia (Malek *et al*., 2011) [12].

3.2 Effect of Extracts on cell Viability

Cell viability was evaluated in the presence of extracts from *Curcuma mangga* rhizomes. Two types of extracts were tested, namely the methanolic extract and the ethyl acetate extract. Increasing concentrations of 10 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml were required for incubation. The viability of cells incubated with the methanolic extract decreased by around 10% at doses of 10 µg/ml, 25 µg/ml and 50 µg/ml compared to cells incubated without extract. However, at a dose of 100 µg/ml, significant cell mortality is observed (figure 6).

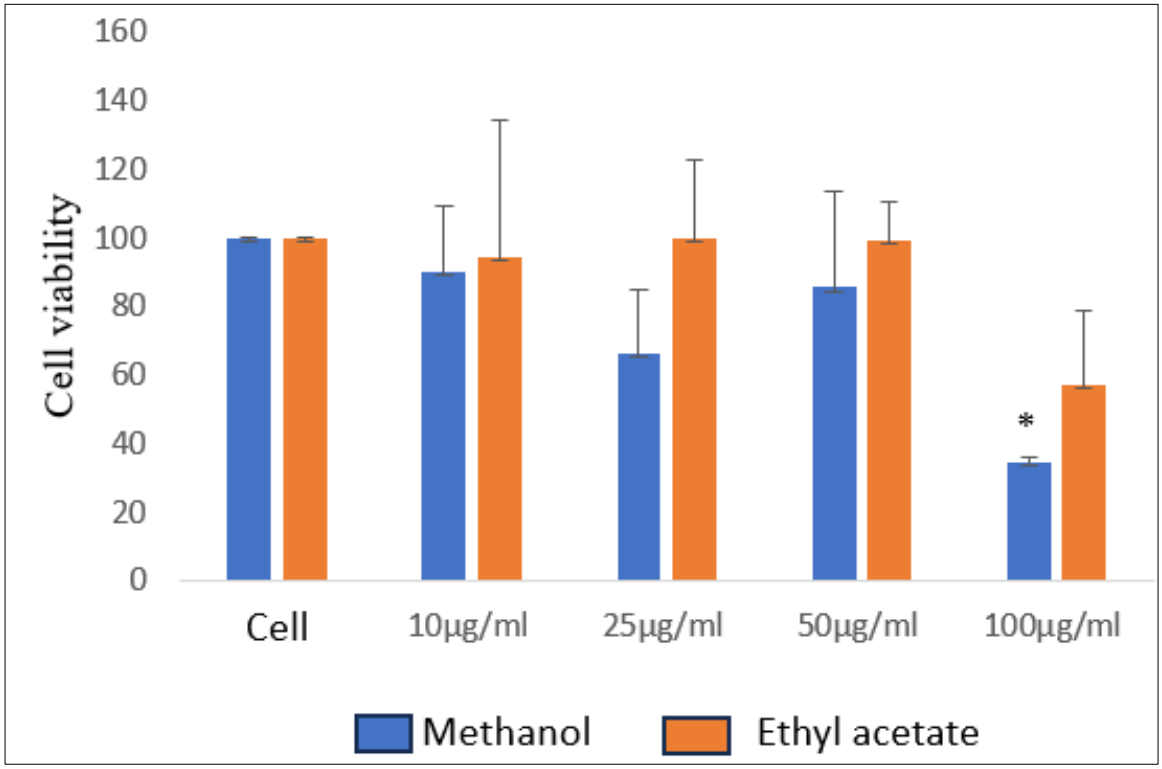

Fig 6: Effect of *Curcuma mangga* extracts on cell viability. * $p< 0.05$

The ethyl acetate extract at concentrations of 10 µg/ml, 25 µg/ml and 50µg/ml have no significant effects on cells. However, mortality is greater at the dose of 100 µg/ml (Figure 6).

3.3 Effect of extracts on ROS production

The anti-inflammatory and antioxidant potential of turmeric mangga extracts was evaluated by their ability to reduce cellular ROS production. Different concentrations of methanolic and ethyl acetate extracts were tested (10, 25, 50 and 100 g/mL). Analysis of the results shows that at a concentration of 100 µg/mL, the extracts significantly inhibit the production of ROS. However, the extract obtained with ethyl acetate is more active, since it inhibits the intracellular production of ROS by up to 63%, compared to the methanolic extract showing an inhibition of 55% (Figure 7).

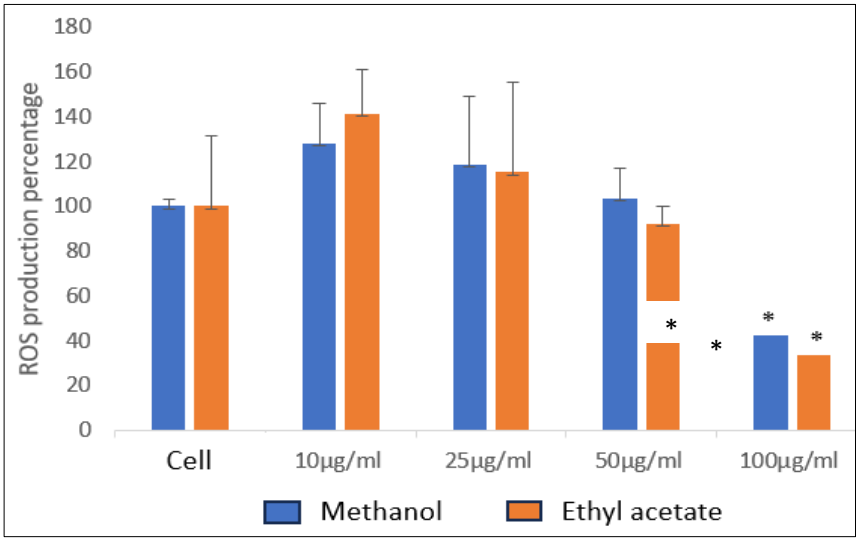

Fig 7: Effect of *Curcuma mangga* extracts on ROS production. * $p< 0.05$

This activity would be linked to the majority compounds identified in the ethyl acetate extract, notably Zerumin A and (E) -labda-8(17), 12-dien-15, 16-dial, and to other compounds not identified but present in this extract. These compounds have already shown their antioxidant (Pujimulyani *et al*., 2010) [15], anti-inflammatory (Ruangsang *et al*., 2010) and cytotoxic (Malek *et al*., 2011; Liu Y *et al*., 2011) [12, 20] potential.

4. Conclusion

The objective of this work focused on the evaluation of the antioxidant potential of *C. mangga* rhizomes. The results showed good inhibition of the production of reactive oxygen species (ROS) by the extracts. At a certain concentration the extracts are non-cytotoxic. Rhizomes of *C. mangga* acclimated to Congo can be used as a source of natural antioxidants.

5. Conflict of Interest

The authors declare no conflict of interest.

6. Acknowledgments

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