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Effect of *Fraxinus angustifolia* (Oleacea) leaf and bark extracts on acute and chronic inflammation: enhanced activity of nanovesicle-trapped extracts

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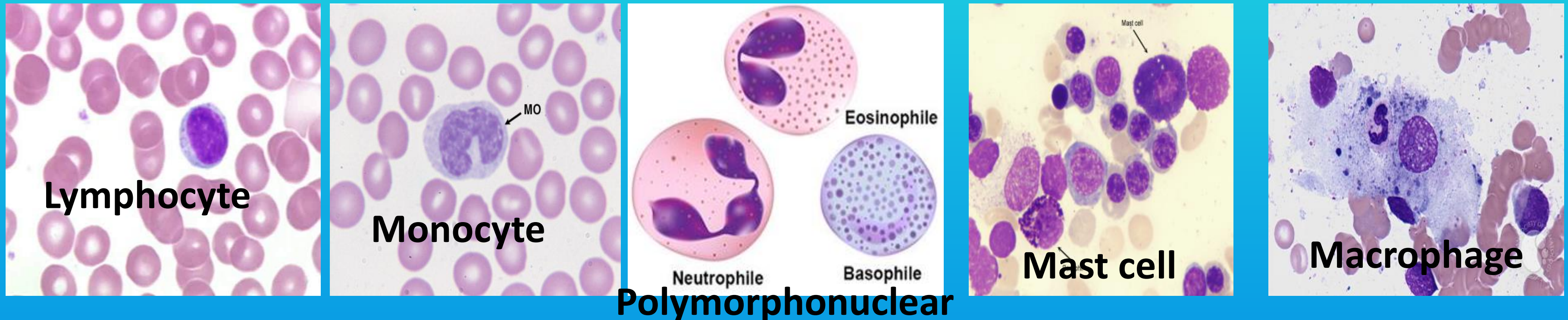
Introduction

- Inflammation is a defense reaction of the organism to various aggressions which can be physical, chemical, biological (immune response) or infectious.
- It is characterized by:

the recruitment of a variety of immune cells in the inflamed site:



the release of various pro-inflammatory cytokines

and

reactive oxygen species / nitrogen (ROS / RNS)

Background

- **Current treatment of inflammation involves NSAIDs and other drugs such as aspirin. Although effective, these drugs have many adverse effects that may hinder their use in the long-term.**

- **Recently, much of the attention has been focused on medicinal plants that have beneficial effects against various diseases; however, more scientific evidence is needed to verify a key role of bioactive components derived from the medicinal plants. In view of these potential health benefits, there has been intensive research on natural phenolic compounds (phenolic acids, flavonoids, and tannins) which are considered to be a major contributor to various biological activities, such as anti-oxidative, anti-inflammatory, anti-cancer and anti-atherosclerotic activities. Recently, we reported anti-oxidative (Atmani et al., 2009) and anti-enzymatic (Berboucha et al., 2010) activities of *Fraxinus angustifolia*.**

- ***Fraxinus angustifolia*** **(ash tree) is widely used in traditional folk medicine; hence, the present investigation was carried out to evaluate the anti-inflammatory and antioxidant potential of extracts and vesicles loaded of *Fraxinus angustifolia* in vitro and in experimental animal models.**

This study was focused on a medicinal plant (*Fraxinus angustifolia*) (family of Oleaceae), widely used in traditional medicine in the treatment of asthma, gout, inflammation, rheumatism and as a diuretic.

Classification

Kingdom: Plantae

Phylum: Spermaphytes

Sub branch: Angiosperms

Class: Dicotyledonous

Subclass: Asteridae

Order: Lamiales

Family: Oleaceae

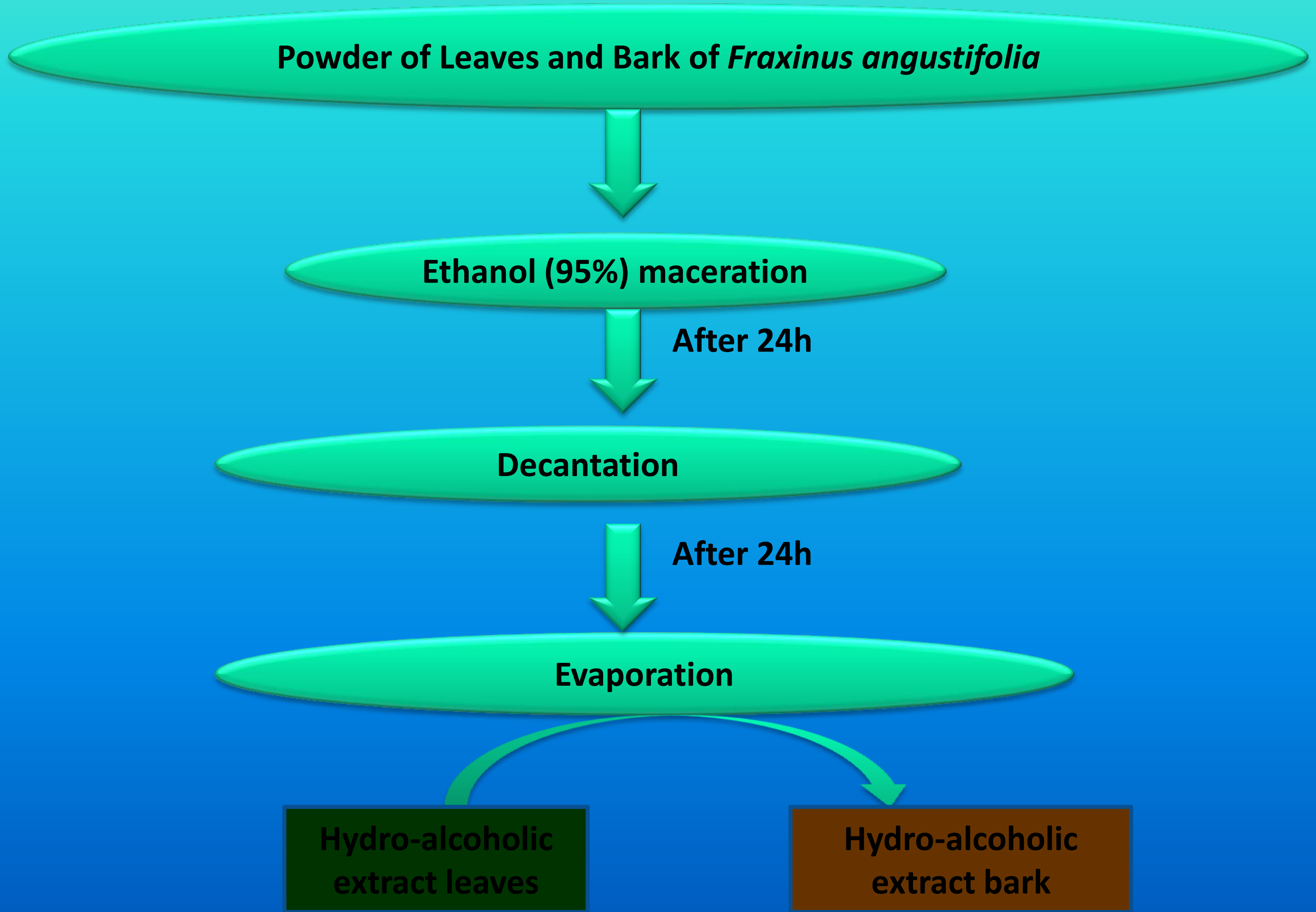
Genus: Fraxinus

Species: *F. angustifolia*

Barks

Leaves

Extraction of phenolic compounds



Carrageenan-induced mouse hind paw oedema

According to the method of Winter et *al*., 1972

Oral Administration of FAB and FAL (150 mg/kg) and indomethacin (10 mg/kg)

After 60 min

Induction of Oedema by subcutaneous injection of carrageenan (0.05 mL of 3% carrageenan suspended in saline) into the right hind paw of mice

Paw volume measurement using a plethysmometer

At 0h, after 1h, 3h and 5h

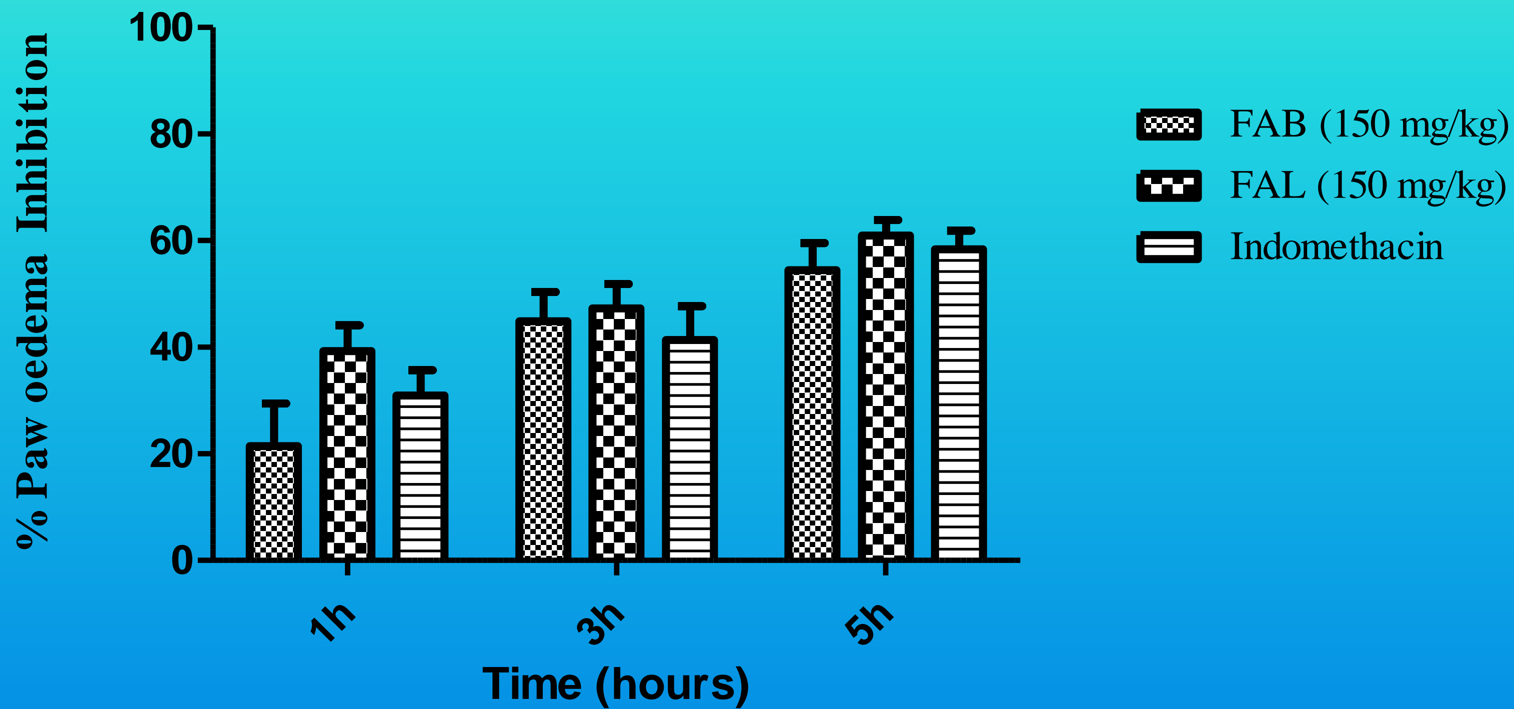


Figure 1: **Percentage of oedema inhibition of *Fraxinus angustifolia* leaf (FAL) and bark (FAB) ethanol extracts (150 mg/kg) on carrageenan-induced mice paw edema, compared to indomethacin (10 mg/kg).**

TPA-induced mouse ear oedema

According to the method of Young and De Young, 1989

Induction of ear oedema by topical application of TPA on the outer and inner surfaces

Application of FAB and FAL extracts (1mg/ear) and indomethacin (0.5 mg/ ear)

Before and After 4h

Measure the thickness of each ear

micrometer

Mouse ear edema induced by multiple topical application of TPA

According to the method of Stanley et *al*., 1991

Chronic inflammation induced by topical application of TPA on both ears of mice on alternate days (1, 3, 5, 8 and 10 days).

After 6h

Administration of extracts twice daily for four consecutive days (8, 9, 10 and 11)

The percentage of swelling inhibition was expressed as the weight difference between ear weight relative to sample a*nd to control group*

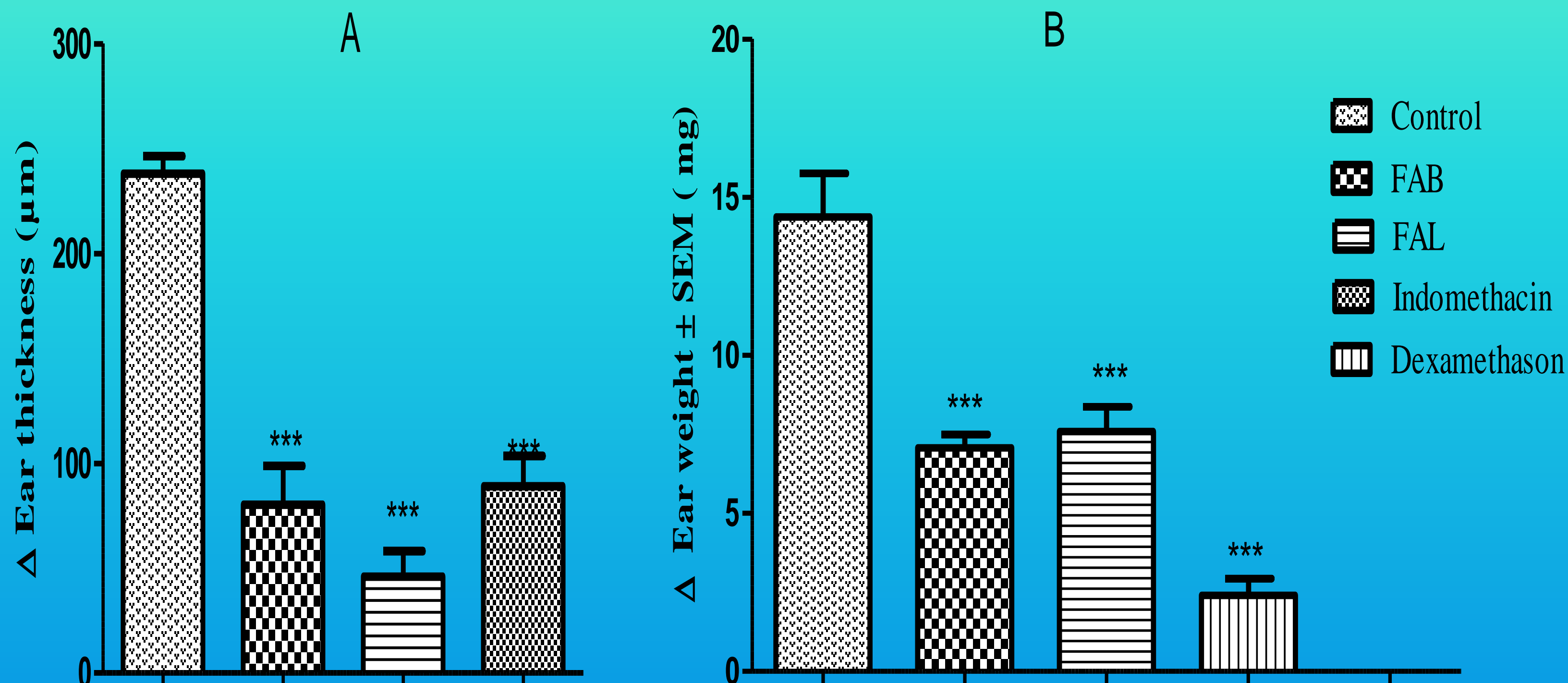


Figure 2: Anti-inflammatory effect of the ethanol extract from *Fraxinus angustifolia* leaves (FAL) and bark (FAB) on acute TPA-induced ear edema (A), ear subchronic inflammation induced by repeated applications of TPA (B) in mice. Statistically significant difference from the control is expressed by * P<*0.001. by Dunnett's multiple comparison test.*

Myeloperoxidase assay

According to the method of De Young et *al*., 1989

Mice ear samples homogenized in cold PBS (80mM, 0.5% HTMB)

Centrifugation for 20 min at 4 °C

30 µl of supernatant add : 100 µL of 80 mM PBS
85 µL of 0.22 M PBS
15 µL of H_2O_2

20 µl of TMB/8%DMF + 30 µl NaOAc

Measure the absorbance at 630 nm

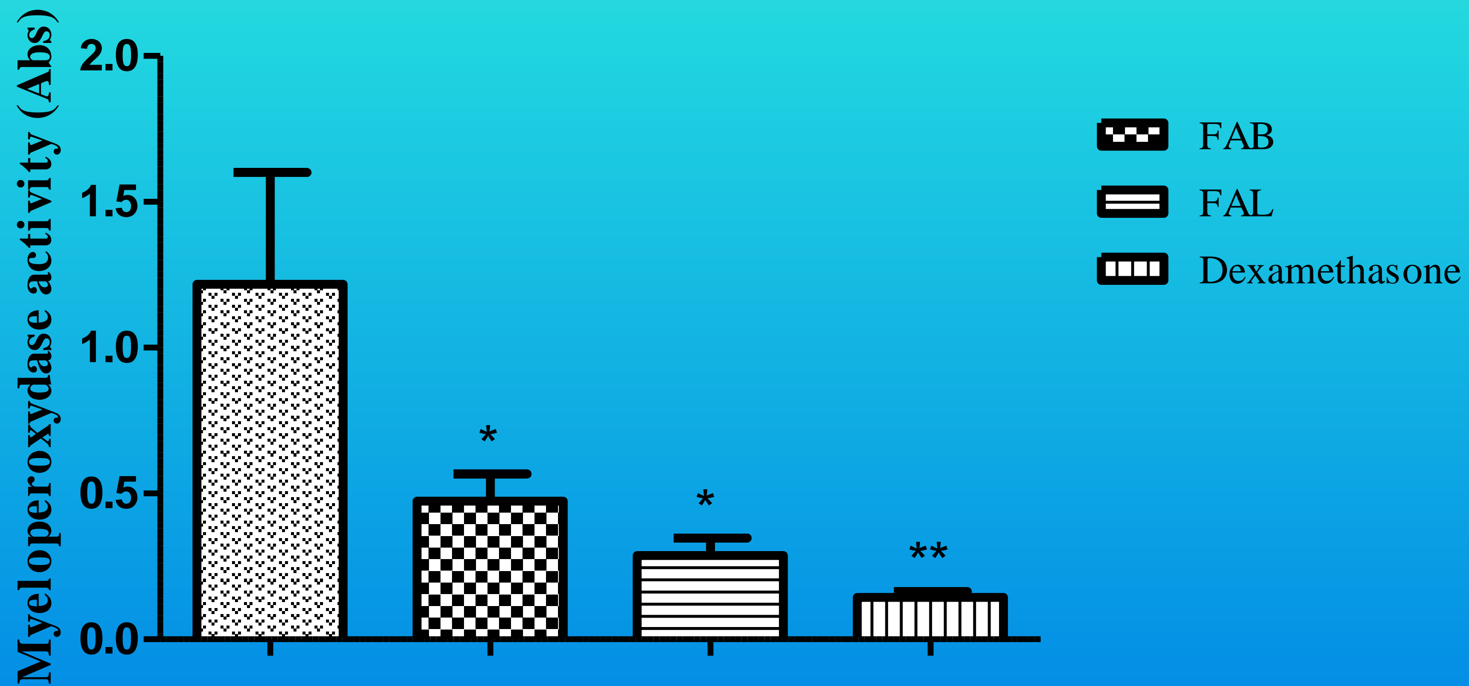
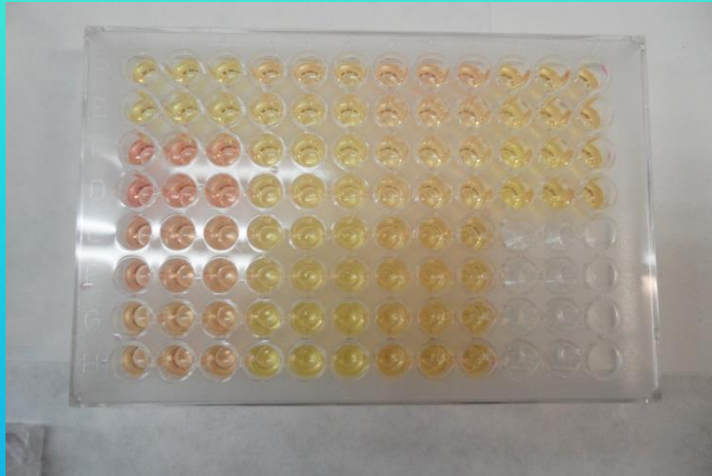


Figure 3: **Effect of FAB and FAL on myeloperoxydase activity** ***P<*0.05 and ***P<0.01. by Dunnett's multiple comparison test.***

Nitric oxide production in RAW 264.7 macrophage



Microplate of nitric oxyde

According to the method of Aquila et *al.*, 2009

Incubate Macrophages (RAW 264.7) in 96 well plates (200 µl/well)

↓ Incubation at 37°C/24h

1µg/µL LPS
Add the extracts dilutions (25,50,75,100µg/ml)

↓

Add 100 µL of the Griess reagent

↓

Measure the absorbance at 540 nm

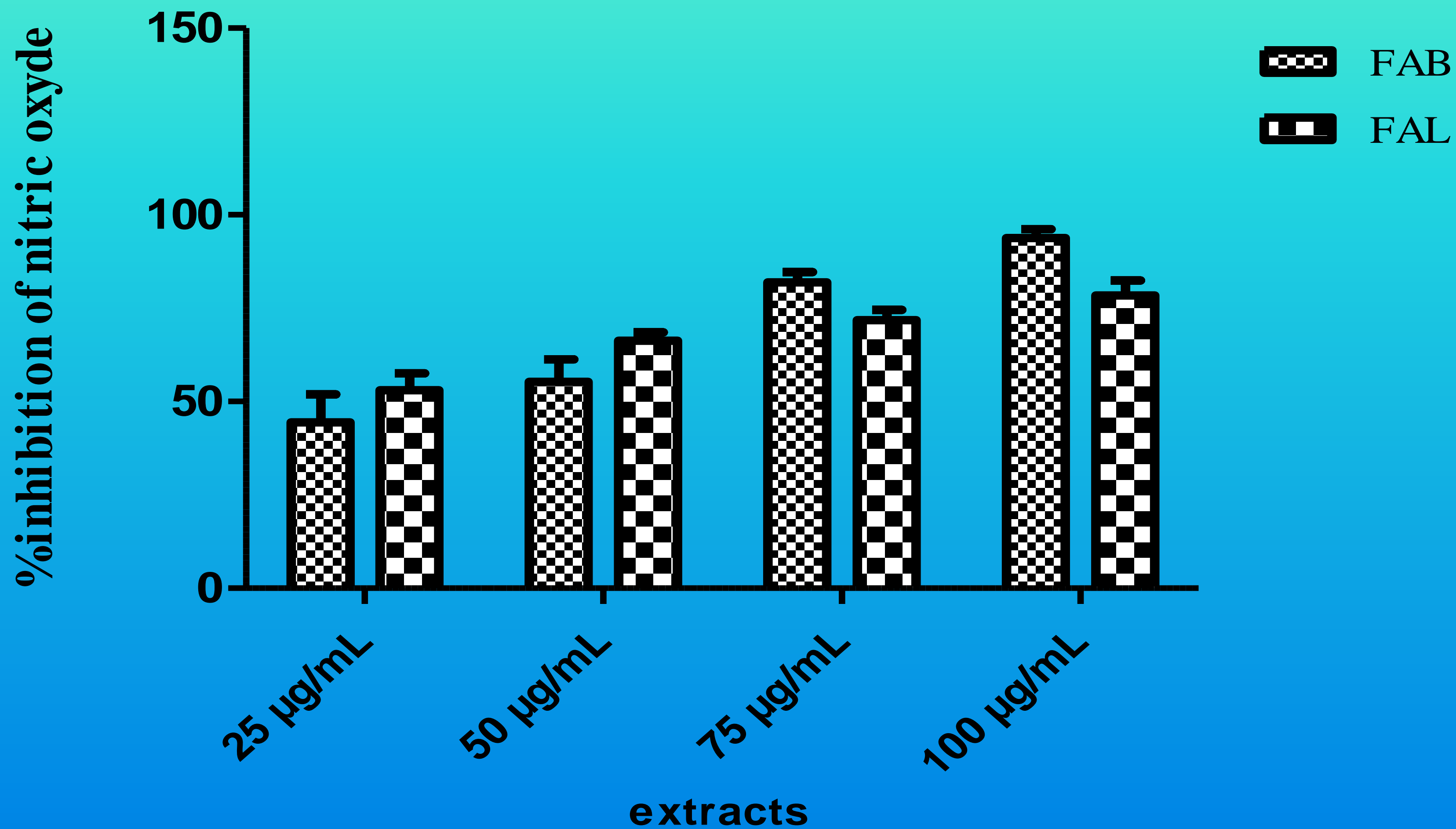


Figure 4: Effect of FAB and FAL on NO production in LPS-stimulated macrophages

Vesicle preparation

leaf and bark of *F. angustifolia* were incorporated in ethosomes and PEVs containing a high amount of water cosolvent (50%,v/v)

Leaf and Bark
-Ethanol/Water
-Ethylene glycol/Water
-Transcutol/Water

60 mg/mL Phospholipon®50

-L ethosomes
-L Trc-PEVs
-L EG-PEVs

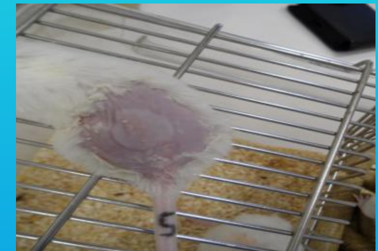
-B ethosomes,
-B Trc-PEVs
-B EG-PEVs

TPA-induced skin inflammation assay

According to the method of Stanley et *al.*, 1991

- Shave the back skin of mice one day before the experiment.

- Apply TPA to the shaved dorsal area to induce cutaneous inflammation (day 1)



- The leaf and bark extract solution or vesicles charged with extracts (ethosomes, Trc-PEVs and EG-PEVs) were topically smeared over the same dorsal site 3 and 6 h after TPA application.

repeat this procedure on days 2 and 3.

- The treated dorsal skin area of mice , weighed to assess any increase indicative of oedema formation.

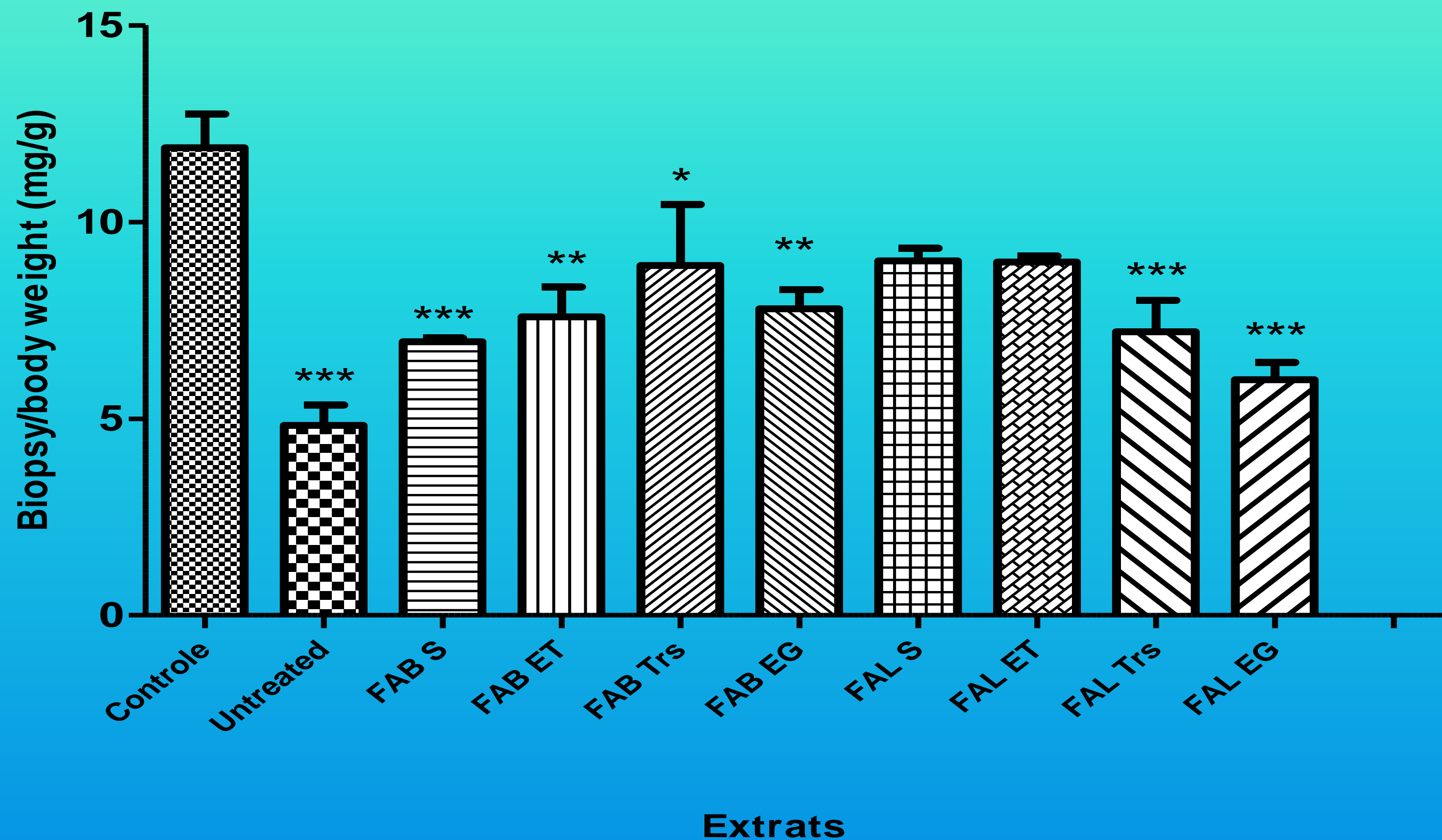


Figure 5: Mouse oedema obtained after TPA treatment followed by application of leaf and bark extract solutions, ethosomes and PEVs. *P<0.05 and *****P*<0.01. * *P*<0.001 by Dunnett's multiple comparison test.***

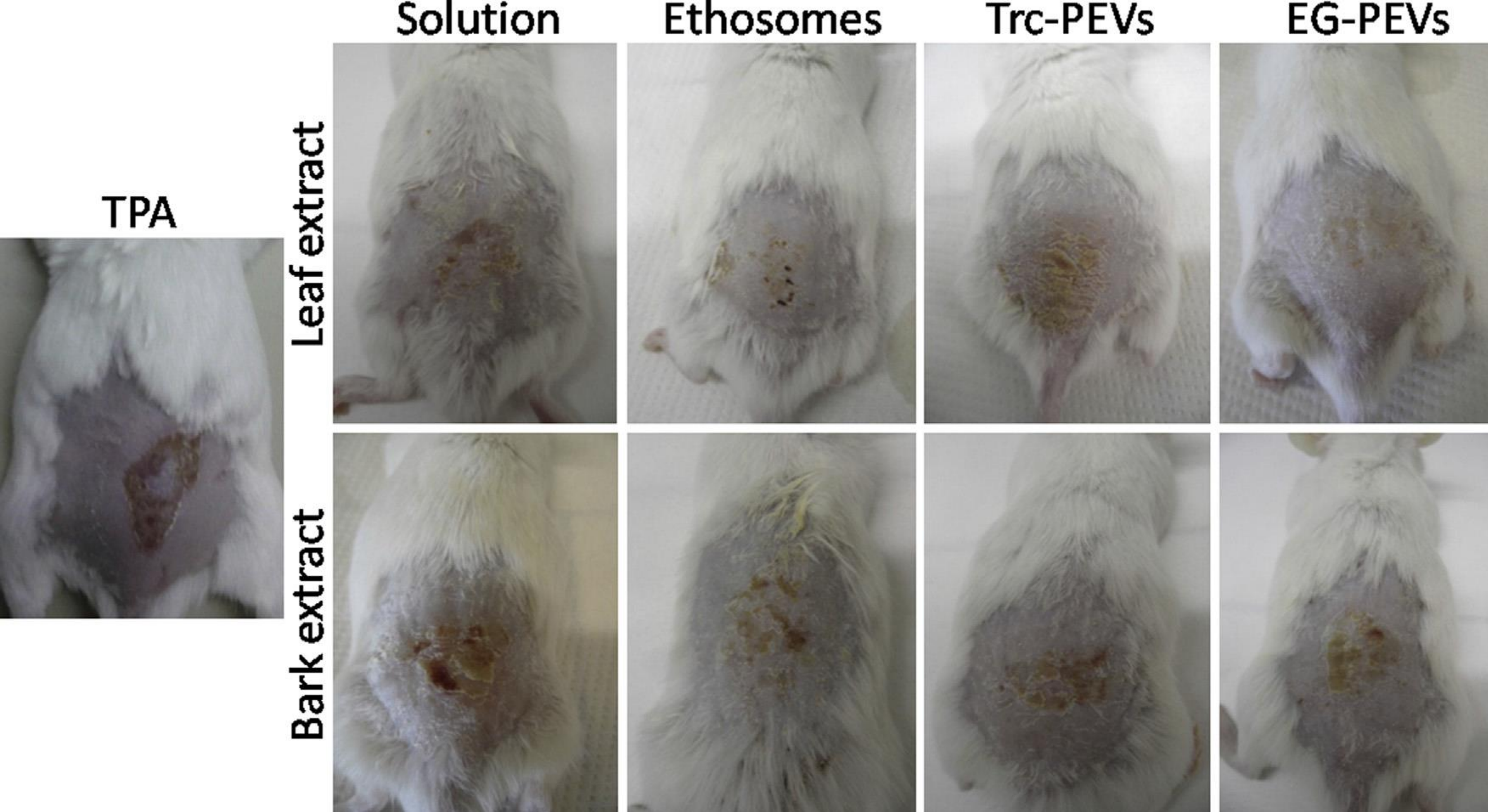


Figure 6: Photographs of dorsal skin of mice treated with TPA to induce inflammation, and treated with TPA followed by the administration of leaf and bark extract solutions, ethosomes and PEVs (Moulaoui et al., Eur. J. Med. Chem (2015).

Myeloperoxidase assay

According to the method of De Young et *al.*, 1989

Mice ear samples homogenized in cold PBS (80mM, 0.5% HTMB)

Centrifugation for 20 min at 4 °C

30 µl of supernatant add : 100 µL of 80 mM PBS
85 µL of 0.22 M PBS
15 µL of H_2O_2

20 µl of TMB/8%DMF + 30 µl NaOAc

Measure the absorbance at 630 nm

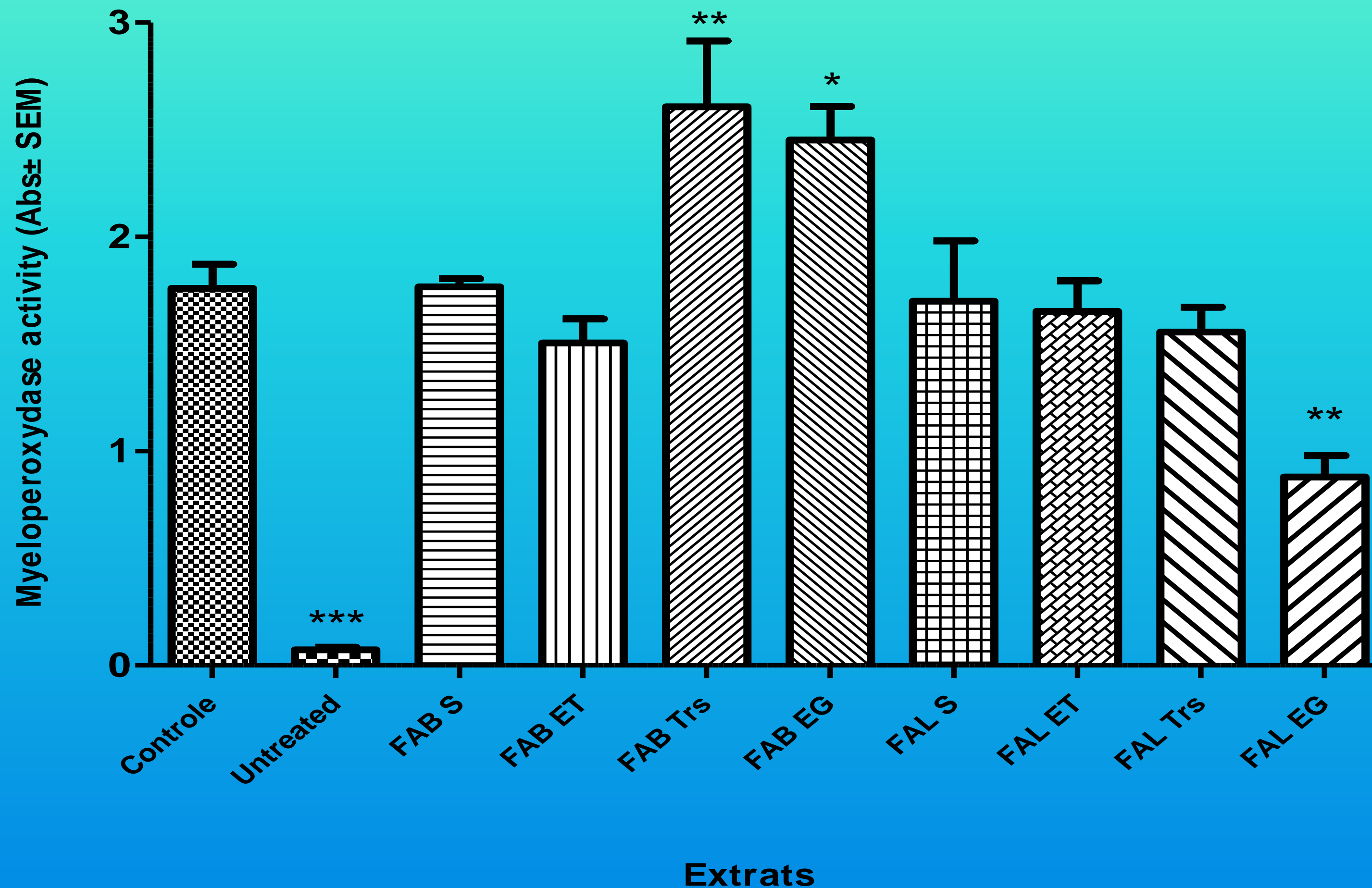


Figure 7 : Absorbance of myeloperoxydase activity after TPA treatment followed by application of leaf and bark extract solutions, ethosomes and PEVs. *$P<0.05$ and **$P<0.01$. by Dunnett's multiple comparison test.

Free radical-scavenging ability by the use of stable ABTS radical

According to the method of Oyedemi et *al.*, 2011

ABTS (7 mM)

Potassium persulfate (2.45 mM)

Solution $ABTS^{\cdot+}$ (Abs= 0.7 nm)

10µl (100 µg/mL solution of extracts of leaf and bark or the three types of the vesicles charged with extracts) + 1ml $ABTS^{\bullet+}$ (7mM, Abs = 0.7nm)

Measure the absorbance at 734 nm

Free radical-scavenging ability by the use of stable DPPH radical

According to the method of Caddeo et *al.*, 2013

20 µL (100 µg/mL solution of extracts of leaf and bark or the three types of the vesicles charged with extracts)

980 µL DPPH methanolic solution (25 mM).

Conservation in the dark for 30 mn

Measure the absorbance at 517 nm

Table 2. Antioxidant activity (%) measured by DPPH, ABTS radical scavenging by leaf and bark extract in ethanolic solutions and vesicles.

		DPPH (%±SD)	ABTS (%±SD)
Leaf extract	Solution	74±2	86±1
	Ethosomes	72±2	88±1
	Trc-PEVs	69±3	86±1
	EG-PEVs	77±3	86±1
Bark extract	Solution	83±1	87±1
	Ethosomes	83±1	87±1
	Trc-PEVs	82±1	87±1
	EG-PEVs	84±1	85±2

Antioxydant activity against oxydative stress in human keratinocytes

According to the method of Phan et *al*., 2001

- Seed keratinocytes in 24 well plates ($5x10^4$ cells/well)

After 24h of incubation at 37 °C

- Add hydrogen peroxide + solution of extracts of leaf and bark or the three types of the vesicles charged with extracts (100µg/mL)

After 4h of incubation at 37 °C

Wash three times with PBS

Add 500 µl MTT

Measure the absorbance at 530nm after 2H

Table 3. **Protective effect of leaf and bark extract ethanolic solutions and vesicles against H_2O_2-induced oxidative stress in human keratinocytes.**

Treatment	Leaf extract (%±SD)	Bark extract (%±SD)
H_2O_2	24±4	24±4
H_2O_2 + Solution	30±7	34±6
H_2O_2 + Ethosomes	76±7	88±9
H_2O_2 + Trc-PEVs	39±7	63±6
H_2O_2 + EG-PEVs	44±3	46±8

HPLC –photodiode array detection system

Extracts

- FAL
- FAB

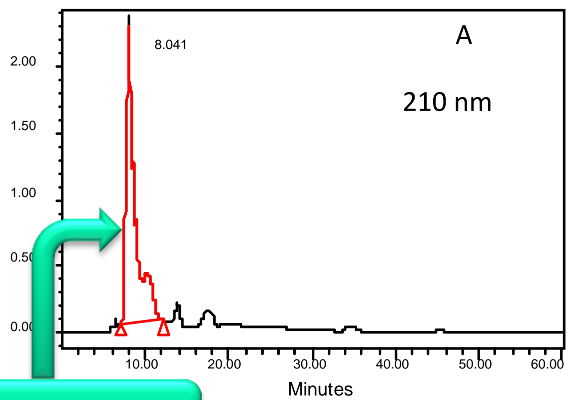
Standards

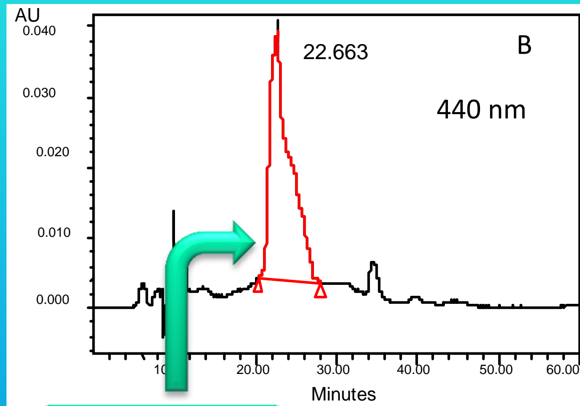
- Rutin
- Quercetin
- Cathenin
- Acid tannic

- ❑ Mobile phase : Mixture of methanol, water, acetonitrile and acetic acid (40:41, 94:18:0,06 v/v)
- ❑ Deliver at a flow rate of 0.3 mL/min.

Identification of major compounds

Major phenolic compounds of FAL





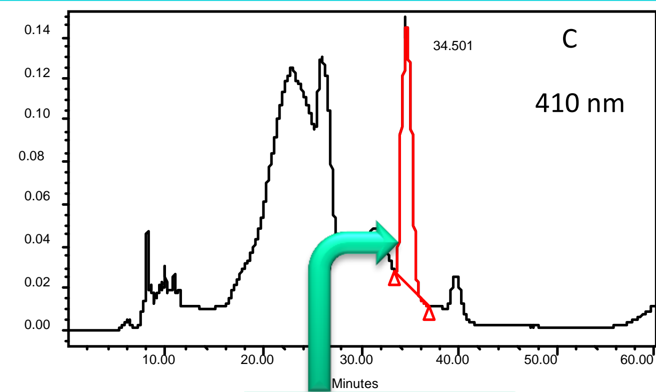


Figure 8: **Chromatograms at different wavelengths of identified components of leaf extract: rutin (A), quercetin (B) and tannic acid (C)**

Major phenolic compounds of FAB

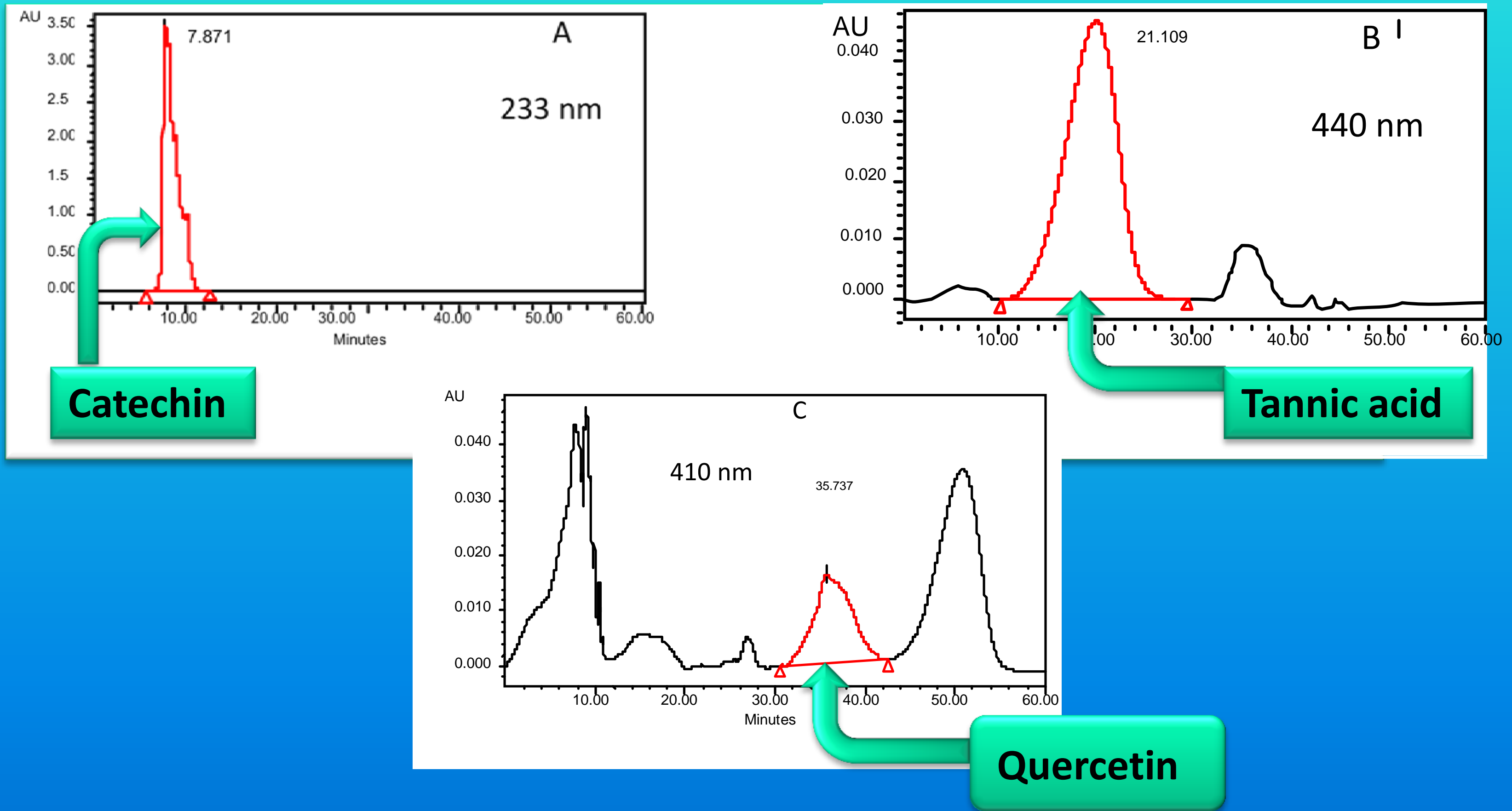


Figure 9: **Chromatograms at different wavelengths of identified components of bark extract: catechin (A), tannic acid (B) and quercetin (C).**

Conclusion

- **Obtained results suggest that the ethanol extract from the leaves and bark of *Fraxinus angustifolia* exhibited interesting anti-inflammatory activity against acute and subchronic oedema, both when orally administered and topically applied.**

- ***Fraxinus angustifolia extracts* showed a significant radical scavenging activity in different in vitro assays.**

- **Hydro-alcoholic extracts of bark and leaves incorporated in nano-vesicles showed significant anti-inflammatory activity, inhibiting the onset of skin wound after TPA treatment. This protective effect was more important in EG-PEVs of leaves formulation, thanks to the excellent ability of the vesicle carrier to increase drug bioavailability in the target tissue.**

- ***Fraxinus angustifolia* may be considered as an important source of polyphenols: rutin, quecetin and tannic acid in the leaves and catechin, quecetin and tannic acid in barks. These compounds are known to be strong free radical scavengers, hydrogen donors and/or metal ion chelators.**

- **Obtained results may justify the use of this plant in folk medicine, although further pharmacological investigations are being carried out in order to fully understand the involvement of phenolic compounds in the anti-inflammatory action.**

Thank you

Let us meet again..

We welcome you all to our future conferences of OMICS International

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June 18-20,2016, Berlin, Germany.

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