

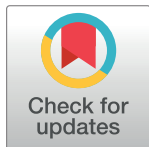
RESEARCH ARTICLE

Antimutagenic, antigenotoxic and antiproliferative activities of *Fraxinus angustifolia* Vahl. leaves and stem bark extracts and their phytochemical composition

Ghania Bouguellid¹, Chiara Russo^{2*}, Margherita Lavorgna², Concetta Piscitelli², Karima Ayouni¹, Erica Wilson³, Hye Kyonn Kim³, Rob Verpoorte³, Young Hae Choi³, Dina Kilani-Atmani¹, Djebbar Atmani¹, Marina Isidori²

1 Laboratoire de Biochimie Appliquée, Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, Bejaia, Algeria, **2** Dipartimento di Scienze e Tecnologie Ambientali, Biologiche e Farmaceutiche, Università della Campania "Luigi Vanvitelli", Caserta, Italy, **3** Natural Products Laboratory, Institute of Biology, Leiden University, Leiden, The Netherlands

* chiara.russo@unicampania.it



OPEN ACCESS

Citation: Bouguellid G, Russo C, Lavorgna M, Piscitelli C, Ayouni K, Wilson E, et al. (2020) Antimutagenic, antigenotoxic and antiproliferative activities of *Fraxinus angustifolia* Vahl. leaves and stem bark extracts and their phytochemical composition. PLoS ONE 15(4): e0230690. <https://doi.org/10.1371/journal.pone.0230690>

Editor: Salvatore V. Pizzo, Duke University School of Medicine, UNITED STATES

Received: December 27, 2019

Accepted: March 5, 2020

Published: April 16, 2020

Copyright: © 2020 Bouguellid et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: This work was supported by the University of Campania "Luigi Vanvitelli" through the VALERE program. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Abstract

In recent years, chronic degenerative diseases such as certain types of cancers, are becoming an evident issue. DNA damage has been for long recognized as a causal factor for cancer development because mutations or chromosomal aberrations affect oncogenes and tumor suppressor genes leading cells to malignant transformation and to the subsequent cancerous growth. Medicinal plants are often used for the prevention or treatment of various diseases with great scientific interest. Among the medicinal plants distributed in the Mediterranean region, *Fraxinus angustifolia* Vahl. has been used in traditional medicine for its remarkable curative properties. However, in spite of this popularity, little works have been performed on the activity so that further studies should be performed to investigate in depth the antimutagenic, antigenotoxic and antiproliferative activities of the plant. Thus, the present study was aimed to the evaluation of the potential antimutagenic, antigenotoxic and antiproliferative properties of leaves and stem bark extracts of this well-known tree. Antimutagenic activity was evaluated by Salmonella mutagenicity assay in *Salmonella typhimurium* TA98 and TA100 strains. The antigenotoxic potential was assessed by *umu* test in the strain of *S. typhimurium* TA1535/pSK1002. Antiproliferative activity was studied on human hepatoblastoma (HepG-2) and on breast adenocarcinoma (MCF-7) cell lines by MTT assay. Furthermore, the antiproliferative activity observed on cancer cells was compared with that on the human normal-like fibroblasts (TelCOFS02MA) and the selectivity index was calculated to understand if extracts were able to exert selective toxicity towards cancer cells. Moreover, phenolic compounds are plant substances with a large spectrum of biochemical activities with antioxidant, antimutagenic and anticarcinogenic effects. Based on the strong evidence of biological activities of phenolic compounds, the study was focused on the determination of total phenolics and flavonoids contents, and the phytochemical composition of the extracts assessed by LC/MS. The ethanol extracts of both leaves and stem barks showed

significant from moderate to strong antimutagenic and antigenotoxic effects. In addition, selective cytotoxicity towards cancer cells was shown by ethanolic leaves extract and aqueous/chloroform leaves and stem bark extracts. The latter showed high levels of total phenolic contents among all the other extracts. Identified phenylethanoids (calceolariosides, verbascoside) and secoiridoids (oleuropein and ligstroside) could be responsible for the demonstrated broad spectrum of healthy properties.

Introduction

Natural products still play a leading role in the treatment of various diseases in diverse forms e.g. extracts, fractions or as a chemical platform. In the history of humanity, plants have always been the most popular source of medicines, mainly thanks to their secondary metabolites with many pharmacological properties. The knowledge of the various healing properties of plants has been transmitted primarily in an empirical way based on folk traditions, and then validated with scientific evidences [1]. At present, one of the hottest topic in medicine is focused on natural bioactive products in the prevention and/or treatment of chronic diseases which have been characterized as the public health challenge of the 21st century. In fact, as explained by Lunenfeld and Stratton [2], in developed countries the rise in healthcare systems and life expectancy, as well as the decrease in fertility rate mainly due to chromosomal abnormalities lead to a rapid increase in the world population aging with consequent chronic degenerative disease increase. In line with the World Health Organization (WHO, 2018), cancer is the second chronic disease (9.6 million deaths in 2018), and cancers of liver (782 000 deaths) and breast (627 000 deaths) are among the most common causes of cancer death. As reported [3], the DNA damage has been long recognized as causal factor for cancer development. In fact, mutations or chromosomal aberrations affect oncogenes and tumor suppressor genes leading cells to malignant transformation. Hence, antimutagenic, antigenotoxic and anticarcinogenic substances play a major role in the primary prevention of cancer development [4, 5].

In recent years, a wide range of medicinal plants and their metabolites have been studied for their potential to decrease the mutagenic and carcinogenic effects of potentially damaging chemicals [6, 7]; in fact, these natural compounds are able to inhibit free radicals and oxidative stress-induced DNA and cellular damages [8, 9].

One of medicinal plants widely distributed in the Mediterranean region is *Fraxinus angustifolia* Vahl a medium-sized deciduous tree, belonging to the Oleaceae family. The plant is extensively used in traditional medicine for its remarkable curative properties. In Algerian folk medicine, different parts of this plant are used to treat many inflammatory diseases [10]; leaves and fruits (samaras) are used in decoctions and infusions as anti-rheumatism, while bark is effective for curing hemorrhoids and fever [11]. Moreover, a mixture composed of powder of samaras, olive oil and honey is used against gonorrhoea [12]. Moreover, an exudate from the bark of *F. angustifolia* Vahl. or *F. ornus* L., called manna, collected in Southern Italy, is used in case of digestive problems [13] as well as expectorant and sedative in cough [14, 15]. When taken in hypertonic solutions, manna acts as a dehydrating agent in the treatment of wounds, ulcers and promotes the flow of the contents of the gall bladder and bile ducts [16, 17].

According to European Medicines Agency (EMA) [18], the biological activities of *F. angustifolia* Vahl. in traditional applications is attributed to the phytochemical constituents; e.g. flavonoids, coumarins, iridoids and secoiridoids having anti-inflammatory and analgesic effects (laxative and diuretic effects might be associated with mannitol). Recently, based on the

chemical structure study [19], phenylethanoids such as calceolariosides (A and B) and verbascoside are the major antioxidants of stem bark extracts of *F. angustifolia* Vahl. Furthermore, verbascoside was also identified in leaves fractions, in addition to secoiridoids (oleuropein and ligstroside) and at least three flavonoid glycosides: kaempferol 3-*O*-rutinoside, isoquercetrin (quercetin 3-*O*-glucoside) and rutin (quercetin 3-*O*- α -L-rhamnopyranosyl-1-6-glucopyranose) which were responsible for leaves antioxidant potential. Furthermore, the latter could also be behind the anti-enzymatic [20–22], antidiabetic, and hepatoprotective [23], as well as wound healing properties [24].

However, to the best of our knowledge, in spite of this popularity, the antimutagenic, antigenotoxic and antiproliferative activities of *F. angustifolia* Vahl leaves and stem bark extracts had never been tested. In this context, the present study aimed to evaluate the antimutagenic, antigenotoxic and antiproliferative properties of *F. angustifolia* Vahl. leaves and stem bark extracts, and to extend the knowledge on their phytochemical composition.

The antimutagenic activity was evaluated by the *Salmonella* mutagenicity assay (Ames test) in *Salmonella typhimurium* TA98 and TA100 strains, auxotrophs for histidine (His⁻). Furthermore, the antigenotoxic potential was assessed by *umu*-test in *S. typhimurium* TA1535/pSK1002. Both antimutagenic and antigenotoxic assays were performed with and without liver homogenate (S9) to find out if metabolizing enzymes were involved in the activation of these natural compounds. Obviously, before beginning antimutagenic and antigenotoxic assays, Ames and *umu* tests were performed also to assess the eventual mutagenicity and genotoxicity of *F. angustifolia* Vahl. extracts.

Antitumor potency was studied by testing *F. angustifolia* Vahl. leaves and stem bark extracts on human hepatoblastoma (HepG-2) and on breast adenocarcinoma (MCF-7) cell lines (two among the most widespread cancers) by MTT assay. Then, to understand if extracts were able to exert selective toxicity towards cancer cells, the antiproliferative activity observed on cancer cells was compared with that of the human normal-like fibroblast cell line (TelCOFS02MA).

Moreover, in order to study the chemical basis of the results obtained, the polyphenolic composition of most active extracts of *F. angustifolia* Vahl. were characterised after fractionation using HPLC/MS method.

Materials and methods

Reagents

Ethanol (CAS: 64-17-5), Ethyl acetate (CAS: 141-78-6) and Chloroform (CAS: 67-66-3) were purchased from Prolabo (Sion, Switzerland). Aroclor 1254-induced male Sprague Dawley rat liver (S9) was purchased from TrinovaBiochem GmbH (Giessen, Germany). Sodium azide (NaN₃, CAS: 26628-22-8) was from JT Baker (Milano, Italy). 2-Nitrofluorene (2-NF, CAS: 607-57-8), 2-amino-anthracene (2-AA, CAS: 613-13-8), 3-methylcholanthrene (3-MC, CAS: 56-49-5), 4-nitroquinoline-1-oxide (4-NQO, CAS: 56-57-5), Cyclophosphamide (CP, CAS: 6055-19-2), L-histidine (CAS: 71-00-1), Biotin (CAS: 58-85-5), and *O*-Nitrophenyl- β -D-galactopyranoside (ONPG, CAS: 369-07-3), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, CAS: 298-93-1) were purchased from Sigma-Aldrich (Milano, Italy). HEPES and Roswell Park Memorial Institute medium (RPMI 1640) were supplied by Lonza BioWhittaker (Verviers, Belgium).

Sample collection and extraction procedure

Fraxinus angustifolia Vahl. leaves (FL) and stem bark (FB) were collected in July 2014 in remote areas of Chemini (963 m altitude, 36°35' latitude and 4°36' longitude), located in the department of Bejaia (Northeastern Algeria). *F. angustifolia* Vahl is not on the list of protected

species, therefore no permission is required to collect it according to Algerian legislation [Art. 4 n° 12–03 of 4/01/2012]. A voucher specimen, with the number (O/n° 59), taxonomically validated by Professor Hacène Abdelkrim, was deposited in the herbarium of Department of Botany, ENSA (Ecole Nationale des Sciences Agronomiques), El-Harrach (Algiers, Algeria).

Leaves and stem bark samples were shade dried at room temperature, then ground into fine powders using an electric blender (KIKA, Labortechnik, Staufen, Germany). Phenolic compounds were extracted following the procedure described [25] and modified [26]. An amount of 100 g of each plant material was macerated in ethanol 96% (1:4 w/v) for 24 h under continuous stirring. After decantation, supernatants were dried using a rotavapor (HEIDOLF). The crude ethanolic extracts (FL1 and FB1, for leaves and stem bark, respectively) were further partitioned using ethyl acetate and distilled water (1:3:1/w:v:v) leading to ethyl acetate fractions (FL2 and FB2) and their respective aqueous fractions (FL3 and FB3). The FL2 and FB2 fractions were then partitioned between chloroform and distilled water (1:3:1/w:v:v) in order to obtain chloroform (FL4 and FB4) and aqueous (FL5 and FB5) fractions.

Salmonella mutagenicity assay (Ames test)

The mutagenic and antimutagenic activities of *F. angustifolia* Vahl. extracts were evaluated by the *Salmonella*/microsome assay, using TA98 and TA100 *S. typhimurium* strains [27, 28]. *Salmonella* strains were from the permanent collection of the Laboratory of Hygiene and Environmental Toxicology, University of Campania, Italy. TA98 strain was used to observe frame-shift mutations, while TA100 was used to assess base-pair substitutions due to missense mutations. Briefly, 100 µL of *S. typhimurium* TA98 or TA100 cultured overnight (10^8 cells), 100 µL of extract at 1000 µg/mL, chosen as the highest concentration for genotoxicity assessment, and 500 µL of phosphate buffer (0.1 M, pH = 7.4), or S9 mixture (lyophilized S9, 1 M NADP, 1 M Glucose-6-phosphate, 0.1 M Phosphate buffer pH 7.4, 0.4 M MgCl₂, 0.4 M KCl) in case of metabolic activation, were added to 2.5 mL of 0.5 M histidine-biotin top agar, and then poured onto minimal glucose agar, in triplicate. Furthermore, 2-NF at 2.5 and 5 µg/mL for TA98, and NaN₃ at 5 and 10 µg/mL for TA100 were used as direct positive controls in absence of metabolic activation. Moreover, 25 and 50 µg/mL of 3-MC for TA98, 50 and 100 µg/mL of CP for TA100 were used as indirect positive controls in presence of metabolic activation. saline solution (0.9% NaCl) was used as negative control. After incubation (37°C/72 h), induced His⁺ revertants were counted and statistically compared to the number of spontaneous revertants on the negative control plate. Plates count after 72h instead of 48h was chosen to facilitate the reading.

The mutagenic ratio (MR) was calculated for each tested extract as the ratio between the average number of revertants per plate of the test compound and the average number of spontaneous revertants per plate of the negative control. A sample was considered mutagenic when a two-fold increase in the number of mutants ($MR \geq 2$) was observed.

For the antimutagenic assay, three different concentrations of the extracts (25, 50 and 100 µg/mL, chosen starting dilutions from the highest concentration of the value 1/10 lower than the concentration used to evaluate the eventual mutagenic effect, 1000 µg/mL) were pre-incubated with direct and indirect mutagens for 2 h at 37°C. The results were expressed as the percentage of the ability of the extracts to inhibit the action of the mutagen, calculated as follows:

$$\text{Inhibition (\%)} = 100 - \left[\left(\frac{T}{M} \right) \cdot 100 \right]$$

Where T is the mean number of revertant colonies in the plate containing both mutagen and test compounds, M is the mean number of revertant colonies in the plate containing only the mutagen [29].

No antimutagenic effect was recorded when inhibition was lower than 25%, a moderate effect for an inhibition value between 25% and 40%, and strong antimutagenicity for values greater than 40% [29].

Umu test

The SOS/*umu* bioassay detects the induction of the SOS-repair system in the *S. typhimurium* TA1535/pSK1002 strain, whose plasmid carries the *umuC:lacZ* fusion gene with the β -galactosidase activity strictly dependent on *umuC* expression in response to specific DNA damaging agents [30]. The *S. typhimurium* TA1535/pSK1002 (optical density of ≥ 800 Formazine Nephelometric Units, FNU) was purchased by EBPI (Burlington, Ontario, Canada) and cultured in tryptone, glucose and ampicillin (TGA)-culture medium at 37°C for 12 h, and then was ten-fold diluted. After 2 h-reincubation, 70 μ L of exponentially growing bacteria (340–350 FNU) were mixed with 180 μ L of the respective extract (1000 μ g/mL), chosen as the highest concentration for genotoxicity assessment, and 20 μ L TGA medium (ten-fold concentrated) into each well of the 96-well microplate, in triplicates. Moreover, saline solution (0.9% NaCl) was used as negative control, 4-NQO (0.05 μ g/mL) and 2-AA (0.2 μ g/mL) were used as positive controls in the absence and presence of S9, respectively. The blank contained 70 μ L of TGA medium instead of the 70 μ L of the bacterial culture. For the determination of indirect genotoxins, 450 μ L of S9 mix (lyophilized S9, 1 M NADP and 1 M Glucose-6-phosphate) were added to 15 mL of bacterial medium. After incubation for 2 h at 37°C under shaking at 150 rpm, 30 μ L from each well were transferred in a new microplate containing fresh medium and re-incubated for a second time. The density of the strain was monitored by measuring the absorbance at 620 nm using a microplate reader (TecanSPECTRAfluor, Männedorf, Switzerland).

For the determination of β -galactosidase activity, 30 μ L of TGA medium was mixed with 120 μ L of B-buffer (0.1 M sodium phosphate pH 7.4, 10 mM KCl, 1 mM MgSO₄, 1 mg/L β -mercaptoethanol, 10 μ L of SDS 1 mg/mL) and the enzymatic reaction was activated by adding 30 μ L of 4.5 mg/mL ONPG. After 30 min of incubation at 28°C, 120 μ L Na₂CO₃ (1M) were added to stop the reaction. The absorbance was recorded at 420 nm.

Genotoxicity was quantified by induction ratio (IR) which is the ratio of absorbance at 420 nm of the sample (T) and of the negative control (N), corrected for growth rate at 620 nm. The sample is considered genotoxic when the induction ratio (IR) is equal to or higher than 1.5, calculated as the β -galactosidase activity of the sample relatively to the negative control:

$$IR = \frac{\beta gal. units_T}{\beta gal. units_N}$$

The β -galactosidase activity (β gal. unit) was calculated as follows:

$$\beta gal. unit = \frac{(A_{420T} - A_{420B})}{(A_{620T} - A_{620B})}$$

Where: A_{420} is the absorbance at 420 nm relative to enzymatic reaction intensity of samples (T) and blank (B); while A_{620} is the absorbance at 620 nm of bacteria growth of samples and blank.

The antigenotoxicity assay was conducted using the same procedure of the genotoxicity assay described above. Three different concentrations of extracts (25, 50 and 100 μ g/mL, chosen starting dilutions from the highest concentration of the value 1/10 lower than the concentration used to evaluate the eventual genotoxic effect, 1000 μ g/mL) were pre-incubated for 2 h at 37°C with known genotoxins, 4-NQO and 2-AA in the absence and presence of metabolic

activation S9, respectively. The percentage of antimutagenicity was calculated [31]:

$$\% \text{Antimutagenicity} = [1 - (\beta\text{gal unit}_{\text{GENOTOXIN+SAMPLE}} / \beta\text{gal unit}_{\text{GENOTOXIN}})] \cdot 100$$

The extract was considered as a strong antigenotoxic when its activity is above 70%, medium when it's between 40 and 70% and neutral when lower than 40% [32].

MTT assay

The antiproliferative activity of *F. angustifolia* Vahl. leaves and stem bark extracts was performed using the tetrazolium dye colorimetric test (MTT assay) [33] on Hep-G2 and MCF-7 cell lines. The cells used were provided by Prof. Abbondanza, Department of Precision Medicine, University of Campania. Periodic quality control testing procedures (authentication, characterization and mycoplasma testing) were performed. The test is based on the activity of mitochondrial reductases that convert tetrazolium salts into formazan, obtaining a purple solution in living cells. Briefly, cells were grown in RPMI1640 medium with 10% fetal bovine serum (FBS), 2% HEPES, 2% L-Glutamine, and 1% penicillin/streptomycin (10,000 U/mL), and were cultured in T-75-cm² tissue culture flasks in humidified atmosphere of 95% air plus 5% CO₂ at 37°C. When the cells were at 80%–90% of confluence; they were collected and counted with the vital dye trypan blue. The cells (1×10⁴/well) were seeded in quadruplicate wells of 96-well plates. After 24 h incubation at 37°C, the medium was removed and replaced with 200 μL of different concentrations (50 to 2000 μg/mL, chosen after range finding tests) of *F. angustifolia* Vahl. leaves and stem bark extracts dissolved in RPMI, chosen after range finding tests. The plates were then incubated for 72 h at 37°C. Each plate had negative control wells containing only the medium. After that, 20 μL of yellow MTT solution (5 mg/mL) was added to each well and the plates were re-incubated for 4 h at 37°C. Hence, the culture medium was removed and 200 μL /well of 2-propanol was used for dissolving the formazan. The absorbance of formazan was measured spectrophotometrically at 590 nm. The results were reported as inhibition cell (IC) percentage:

$$\text{IC}(\%) = 1 - \left[\frac{\text{OD}_{590} \text{ sample}}{\text{OD}_{590} \text{ negative control}} \right] \cdot 100$$

Independent experiments were pooled and statistically analysed by Prism 5.03 version (GraphPad Inc., San Diego, CA, USA). Thus, IC₅₀ values were calculated by non-linear concentration/response regression model.

Furthermore, selectivity index (SI) was calculated [34] to compare the antiproliferative activity observed on cancer cells with that of the human normal-like fibroblast cell line (Tel-COFS02MA, ATCC® CRL 4005™ purchased from American Type Culture Collection, Milan, Italy). A selective toxicity towards cancer cells occurs when SI value is greater than 2. SI value was calculated using the formula given below:

$$\text{SI} = \frac{\text{IC}_{50} \text{ normal like cells}}{\text{IC}_{50} \text{ cancer cells}}$$

Determination of total phenolics and flavonoids contents

The total phenolics contents of *F. angustifolia* Vahl. extracts were quantified by the Folin-Ciocalteu method and the amounts of phenolics were calculated from catechin standard curve [35]. Flavonoids contents were determined by the aluminium chloride procedure [36] and deduced from a rutin standard curve to be expressed as μg of catechin equivalent/mg of dry extract.

UHPLC/MS investigation

The LC-MS analyses were carried out on the apparatus consisting of the following elements: Dionex Ultimate 3000 UHPLC system equipped with a quaternary pump (RS pump), an auto-sampler (RS autosampler) and UV detector with diode arrays (PE 785 A) linked to a high-resolution triple-quadrupole mass spectrometer (Bruker micro OTOF-QII, Bremen, Germany), equipped with an electrospray ionisation interface. The ethanolic extracts and their respective fractions of leaves and stem barks of *F. angustifolia* Vahl. (1 mg/mL) were injected onto a C18 Symetry HPLC column (Waters) (3.5 μ m, 75 x 4.6 mm). The separations were carried out at room temperature with a mobile phase consisting of two water-acid solvents. Formic acid 0.1% (A) and methanol-0.1% formic acid (B) under the following conditions: 0 to 15 min, 13% B for 15 min, then in 5 min a linear gradient of 55 to 85% B then 85% B for 1 min, finally, return to the initial conditions (15% B) in 2 minutes to rebalance the column before any new injection. For all analyses, the solvents used are of HPLC quality, the flow rate was set at 0.350 mL/min, and the measurement wavelengths were set at 280, 360 and 450 nm. For comparative purposes, the injection volume and the concentrations of the injected solutions were fixed at 5 μ L and 1 mg/mL, respectively, for all injected extracts and fractions.

Statistical analysis

Tests were from three independent experiments. The results were expressed as mean and standard deviation. Analysis of variance (one-way ANOVA model) was performed in combination with *Dunnett's post-test* for multiple comparisons. Differences from the controls were considered significant as * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$. GraphPad Prism 5.03 version software was used for all statistical assessments.

Results

Mutagenicity/genotoxicity

Before the assessment of the protective effects of *F. angustifolia* Vahl. extracts, Ames and *umu* tests were also used to study, respectively, the eventual mutagenic and genotoxic effects of these extracts. At 1000 μ g/mL, they did not reveal any mutagenic effect, as reported in [S1](#) and [S2](#) Figs. In fact, in *S. typhimurium* TA 98 and TA 100 strains, there were observed neither direct/indirect frame-shift mutations nor direct/indirect missense mutations ($M R < 2$) were observed. Similarly, no direct/indirect genotoxic effects were observed ([S1 Table](#)) indicating no induction of the SOS-repair system in the *S. typhimurium* TA1535/pSK1002 strain ($IR < 1.5$).

Antimutagenicity

Results of antimutagenic activity with and without metabolic activation are presented respectively in [Tables 1](#) and [2](#), as the mean of revertants/plates \pm SD of three independent experiments (each one based on three replicates). Inhibition rate percentage was calculated [[29](#)]. Basically, significant moderate and strong antimutagenic effects exerted by *F.angustifolia* Vahl. leaves and stem bark extracts were observed in *S. typhimurium* strains. Three concentrations (25, 50 and 100 μ g/mL) of extracts were co-incubated for 72h with standard direct mutagens. Altogether, inhibition rate percentage values, calculated for TA100, were higher than those of TA98 ([Table 1](#)).

Thus, extracts were mainly able to inhibit the direct mis-sense mutations caused by both concentrations of NaN_3 (5 and 10 μ g/mL) in TA 100, but were less active in inhibiting direct frame-shift mutations towards the higher concentration of 2-NIT (5 μ g/mL) in TA 98. Specifically, FL2, FL4, FB1, FB2, FB4 did not induce significant inhibition of the frame-shift

Table 1. Direct antimutagenic activities of *F. angustifolia* Vahl. Antimutagenic activities of *F. angustifolia* Vahl. leaves and stem bark extracts on TA98 and TA100 strains after 72h co-incubation with direct mutagens, respectively 2-NF (2.5 and 5 µg/mL) and NaN₃ (5 and 10 µg/mL), in the absence of metabolic activation system S9.

Treatment [µg/mL]		TA 98 revertants/plate (mean±SD)		Inhibition rate (%)		TA 100 revertants/plate (mean±SD)		Inhibition rate (%)	
		2-NF 2.5µg/mL	2-NF 5µg/mL	2-NF 2.5µg/mL	2–5µg/mL	NaN ₃ 5µg/	mLNaN ₃ 10µg/mL	NaN ₃ 5µg/mL	NaN ₃ 10µg/mL
FL1	25	77 ± 4 ***	183 ± 79*	52 ^c	46 ^c	237 ± 25 ***	283 ± 50 ***	49 ^c	56 ^c
	50	88 ± 16 ***	163 ± 59 *	46 ^c	52 ^c	282 ± 20 ***	345 ± 7 ***	39 ^b	47 ^c
	100	99 ± 10 ***	233 ± 3	39 ^b	32 ^b	250 ± 3 ***	354 ± 8 ***	46 ^c	45 ^c
FL2	25	94 ± 17 ***	333 ± 22	42 ^c	2 ^a	252 ± 19 ***	381 ± 14 ***	45 ^c	41 ^c
	50	96 ± 8 ***	330 ± 44	41 ^c	3 ^a	222 ± 6 ***	379 ± 13 ***	52 ^c	41 ^c
	100	98 ± 16***	336 ± 11	40 ^b	1 ^a	213 ± 21 ***	352 ± 18 ***	54 ^c	46 ^c
FL3	25	105 ± 3**	175 ± 72 *	35 ^b	49 ^c	261 ± 27 ***	306 ± 6 ***	43 ^c	53 ^c
	50	95 ± 9 ***	324 ± 62	41 ^c	5 ^a	242 ± 59 ***	302 ± 14 ***	48 ^c	53 ^c
	100	98 ± 10 ***	240 ± 14	40 ^b	30 ^b	267 ± 4 ***	348 ± 40 ***	42 ^c	46 ^c
FL4	25	77 ± 4 ***	210 ± 13	52 ^c	38 ^b	290 ± 18 ***	339 ± 44 ***	37 ^b	48 ^c
	50	86 ± 16 ***	279 ± 13	47 ^c	18 ^a	349 ± 92	388 ± 25 ***	24 ^a	40 ^b
	100	88 ± 4 ***	273 ± 16	46 ^c	20 ^a	252 ± 17 ***	382 ± 48 ***	45 ^c	41 ^c
FL5	25	96 ± 7 ***	330 ± 17	41 ^c	3 ^a	277 ± 73 ***	301 ± 40 ***	40 ^b	53 ^c
	50	98 ± 17 ***	182 ± 73 *	40 ^b	47 ^c	374 ± 9	304 ± 6 ***	19 ^a	53 ^c
	100	111 ± 9 **	256 ± 40	31 ^b	25 ^b	327 ± 16 **	308 ± 7 ***	29 ^b	52 ^c
FB1	25	81 ± 4 ***	193 ± 80	50 ^c	43 ^c	225 ± 23 ***	368 ± 5 ***	51 ^c	43 ^c
	50	102 ± 17 **	332 ± 14	37 ^b	3 ^a	258 ± 8***	367 ± 4 ***	44 ^c	43 ^c
	100	89 ± 11 ***	240 ± 28	45 ^c	30 ^b	296 ± 19***	354 ± 3 ***	36 ^b	45 ^c
FB2	25	71 ± 13 ***	306 ± 8	56 ^c	10 ^a	248 ± 11 ***	365 ± 17 ***	46 ^c	44 ^c
	50	108 ± 17 *	332 ± 6	33 ^b	3 ^a	244 ± 34 ***	314 ± 42 ***	47 ^c	51 ^c
	100	83 ± 16 ***	334 ± 0	49 ^c	2 ^a	264 ± 23 ***	300 ± 96 ***	43 ^c	54 ^c
FB3	25	80 ± 4 ***	218 ± 10	51 ^c	36 ^b	274 ± 41 ***	314 ± 4 ***	41 ^c	51 ^c
	50	106 ± 6 *	173 ± 71 *	35 ^b	33 ^b	250 ± 5 ***	372 ± 17 ***	46 ^c	43 ^c
	100	75 ± 4 ***	230 ± 19	54 ^c	37 ^b	262 ± 2 ***	350 ± 14 ***	43 ^c	46 ^c
FB4	25	109 ± 29 *	323 ± 10	33 ^b	5 ^a	258 ± 47 ***	358 ± 7 ***	44 ^c	45 ^c
	50	105 ± 9 **	191 ± 84	35 ^b	44 ^c	298 ± 60 ***	348 ± 17 ***	35 ^b	46 ^c
	100	76 ± 8 ***	271 ± 58	53 ^c	21 ^a	366 ± 16	355 ± 33 ***	21 ^a	45 ^c
FB5	25	103 ± 27 **	279 ± 66	36 ^b	18 ^a	292 ± 6 ***	351 ± 51 ***	37 ^b	46 ^c
	50	110 ± 6 *	211 ± 86	32 ^b	38 ^b	276 ± 11 ***	326 ± 20 ***	40 ^b	50 ^c
	100	113 ± 6 *	154 ± 54**	30 ^b	55 ^c	306 ± 89 ***	334 ± 20 ***	34 ^b	48 ^c
M		162±44	341±79	-	-	461±43	647±16	-	-
NC		60 ± 7		-	-	220±25		-	-

Results are expressed as mean of revertants/plates ± SD (three independent experiments). Inhibition rate percentage was calculated as follows: $100 - [(T/M) \times 100]$ where T is the mean number of revertant colonies in the plate containing both mutagen and tested extract, and M is the mean number of revertant colonies in the plate containing only the mutagen [29]. Significant difference for *p < 0.05, **p < 0.01, *** p < 0.001 (Dunnett's test) was calculated comparing extracts co-treated with standard mutagens to single standard mutagens.

NC negative control; M mutagen; 2-NF 2- nitrofluorene; NaN₃ Sodium Azide FL *F.angustifolia* Vahl. leaves; FB *F.angustifolia* Vahl. stem bark

1 Ethanolic; 2 Organic/Ethyl Acetat; 3 Aqueous/ Ethyl Acetat; 4 Organic/ Chloroform; 5 Aqueous/ Chloroform

^aNo antimutagenic effect (< 25% inhibition)

^bModerate effect (25%– 40% inhibition)

^cStrong antimutagenic effect (> 40% inhibition) [29].

<https://doi.org/10.1371/journal.pone.0230690.t001>

mutations induced by 2-NIT (5 µg/mL). Indirect antimutagenicity experiments, were performed only at 25 µg/mL of extracts, which was the lowest effective concentration obtained in direct antimutagenicity experiments. Significant moderate and strong antimutagenic effects

Table 2. Indirect antimutagenic effects of *F. angustifolia* Vahl. Antimutagenic effects of *F. angustifolia* Vahl. leaves and stem bark extracts on strains TA98 and TA100 after 72h co-incubation with indirect mutagens, respectively 3-MC (25 and 50 µg/mL) and CP (50 and 100 µg/mL), in presence of metabolic activation system S9.

Treatments 25µg/mL	TA 98				TA 100			
	Mean revertants/plate±SD		Inhibition rate (% mean±SD)		mean revertants/plate±SD		Inhibition rate (% mean ±SD) ^b	
	3-MC 25µg/mL	3-MC 50µg/mL	3-MC 25µg/mL	3-MC 50µg/mL	CP 50µg/mL	CP 100µg/mL	CP 50µg/mL	CP 100µg/mL
FL1	79 ± 5 ***	158 ± 1 ***	40 ± 4 ^b	32 ± 3 ^b	244 ± 13 ***	484 ± 13 ***	43 ± 5 ^c	29 ± 3 ^b
FL2	87 ± 2 ***	173 ± 6 ***	34 ± 3 ^b	26 ± 4 ^b	226 ± 5 ***	439 ± 23 ***	47 ± 4 ^c	35 ± 4 ^b
FL3	89 ± 2 ***	190 ± 2 ***	32 ± 3 ^b	18 ± 4 ^a	251 ± 8 ***	458 ± 2 ***	41 ± 5 ^c	32 ± 2 ^b
FL4	76 ± 2 ***	155 ± 4 ***	42 ± 3 ^c	33 ± 3 ^b	300 ± 16 ***	467 ± 7 ***	29 ± 6 ^c	31 ± 2 ^b
FL5	76 ± 0 ***	151 ± 6 ***	42 ± 2 ^c	35 ± 4 ^b	252 ± 7 ***	455 ± 4 ***	41 ± 5 ^c	33 ± 2 ^b
FB1	84 ± 2 ***	155 ± 2 ***	36 ± 3 ^b	33 ± 3 ^b	263 ± 13 ***	460 ± 13 ***	38 ± 5 ^c	32 ± 3 ^b
FB2	75 ± 1 ***	152 ± 15 ***	43 ± 2 ^c	35 ± 7 ^b	240 ± 6 ***	506 ± 30 ***	44 ± 4 ^c	25 ± 5 ^b
FB3	84 ± 1 ***	171 ± 1 ***	36 ± 3 ^b	27 ± 3 ^b	231 ± 6 ***	490 ± 18 ***	46 ± 4 ^c	28 ± 3 ^b
FB4	88 ± 2 ***	168 ± 2 ***	33 ± 3 ^b	28 ± 3 ^b	235 ± 6 ***	443 ± 18 ***	45 ± 4 ^c	35 ± 3 ^b
FB5	80 ± 5 ***	160 ± 2 ***	39 ± 4 ^b	31 ± 3 ^b	229 ± 12 ***	436 ± 21 ***	46 ± 5 ^c	36 ± 4 ^c
M	131 ± 5	233 ± 10	-	-	425 ± 31	677 ± 18	-	-
NC	60 ± 7	-	-	-	220 ± 25	-	-	-

Results are expressed as mean of revertants/plates ± SD (three independent experiments). Inhibition rate percentage was calculated as follows: $100 - [(T/M) \times 100]$ where T is the mean number of revertant colonies in the plate containing both mutagen and tested extract, and M is the mean number of revertant colonies in the plate containing only the mutagen [25]. Significant difference for *** $p < 0.001$ (Dunnett's test) was calculated comparing extracts co-treated with standard mutagens to single standard mutagens.

NC negative control; M mutagen; 3MC 3-Metilcolanthrene; CP Cyclophosphamide; FL *F.angustifolia* Vahl. leaves; FB *F.angustifolia* Vahl. stem bark
1 Ethanolic; 2 Organic/Ethyl Acetat; 3 Aqueous/ Ethyl Acetat; 4 Organic/ Chloroform; 5 Aqueous/ Chloroform

^aNo antimutagenic effect (< 25% inhibition)

^bModerate effect (25%– 40% inhibition)

^cStrong antimutagenic effect (> 40% inhibition) [29].

<https://doi.org/10.1371/journal.pone.0230690.t002>

(*** $p < 0.0001$) were observed for all extracts in presence of metabolic activation (Table 2). Hence, protective effect towards indirect frame- shift and missense mutations was exhibited, respectively in both TA98 and TA100, at both concentrations of standard indirect mutagens (3-MC: 25 and 50 µg/mL; CP: 50 and 100 µg/mL).

Antigenotoxicity

Antigenotoxicity results, due to the properties of the extracts in inhibiting the SOS response in *S. typhimurium* TA1535/pSK1002, were expressed as mean of induction ratio (IR) ± SD ($n = 3$), and are reported in Table 3. Significant differences (** $p < 0.001$, *** $p < 0.0001$) were observed between IR values obtained from extracts co-treated with direct standard genotoxin (4-NQO, 0.05 µg/mL) and IR values obtained from the single 4-NQO. Antigenotoxicity (% mean ± SD), calculated [31], revealed a moderate or strong effect induced by all extracts towards the direct genotoxin in absence of metabolic activation, with the highest inhibition by FL5 and FB5 (antigenotoxicity by 70%). On the contrary, in presence of metabolic activation, FL3, FB4 and FB5 showed a neutral effect (Antigenotoxicity less than 40%), while FL1, FL2, FL4 and FB1 showed significant (** $p < 0.001$, *** $p < 0.0001$) moderate to strong protective effect against the indirect genotoxin (2AA, 0.2 µg/mL).

Antiproliferative activity

Results regarding the potential antiproliferative activity exerted after 72h (MTT assay) by *F. angustifolia* Vahl. extracts on Hep-G2 and MCF-7 cell lines were expressed as IC₅₀ values and

Table 3. Antigenotoxicity of *F. angustifolia* Vahl. Antigenotoxicity of *F. angustifolia* Vahl. leaves and stem bark extracts (25, 50, 100 µg/mL) after 2h co-incubation with standard genotoxins: 4-NQO 0.05µg/mL and 2-AA 0.20µg/mL, respectively for the treatment in absence and in presence of metabolic activation S9.

Treatment [µg/mL]		IR ± SD			
		-S9		+S9	
NC	0	1.00 ± 0.00		1.00 ± 0.00	
4-NQO	0.05	3.13 ± 0.34		-	
2-AA	0.20	-		4.56 ± 0.66	
		+ 4-NQO		+ 2-AA	
		IR ± SD	Antigenotoxicity (% mean ± SD)	IR ± SD	Antigenotoxicity (% mean ± SD)
FL1	25	1.60±0.24***	48.90 ± 0.29 ^b	2.09± 0.36**	54.00 ± 1.72 ^b
	50	1.56±0.17***	50.10 ± 2.25 ^b	2.46± 0.42	45.73 ± 1.93 ^b
	100	1.82±0.30**	42.05 ± 0.68 ^b	2.35± 0.33*	48.20 ± 3.34 ^b
FL2	25	1.13±0.04***	63.50 ± 4.55 ^b	1.60± 0.48***	65.29 ± 3.40 ^b
	50	1.10±0.10***	64.63 ± 2.00 ^b	1.77± 0.35**	61.20±0.34 ^b
	100	1.12±0.22***	64.22 ± 1.56 ^b	1.57± 0.35***	65.56 ± 0.52 ^b
FL3	25	1.75±0.47**	44.55 ± 6.50 ^b	2.92± 0.40	35.55 ± 4.67 ^a
	50	1.69±0.21**	46.05 ± 1.55 ^b	3.25± 0.74	28.95 ± 1.59 ^a
	100	1.74±0.32**	44.47 ± 1.69 ^b	2.60± 0.13	41.93 ± 9.10 ^b
FL4	25	1.24±0.23***	60.41 ± 1.25 ^b	1.87± 0.68**	59.60 ± 6.65 ^b
	50	1.15±0.03***	62.66 ± 6.78 ^b	2.08± 0.71**	55.10 ± 6.23 ^b
	100	1.23±0.13***	60.64 ± 2.39 ^b	1.64± 0.38***	64.16 ± 0.95 ^b
FL5	25	0.92±0.03***	70.31 ± 5.56 ^c	1.92± 0.23**	57.44 ± 3.73 ^b
	50	1.13±0.02***	63.50 ± 4.94 ^b	2.28 ± 0.10*	49.17 ± 8.23 ^b
	100	1.03±0.04***	66.84 ± 3.80 ^b	2.47± 0.38	45.52 ± 3.00 ^b
FB1	25	1.37±0.50***	57.02 ± 9.30 ^b	2.26± 0.82*	51.16 ± 7.96 ^b
	50	1.38±0.33***	56.21 ± 3.87 ^b	2.50± 0.88	46.02 ± 8.08 ^b
	100	1.41±0.32***	55.09±3.27 ^b	1.89± 0.40**	58.49 ± 0.16 ^b
FB2	25	1.19±0.26***	62.13 ± 2.45 ^b	2.47± 0.30	45.31 ± 4.72 ^b
	50	1.23±0.18***	60.69 ± 0.21 ^b	2.27± 0.06*	49.35 ± 9.25 ^b
	100	1.43±0.24***	54.35±0.67 ^b	2.66± 0.16	40.72 ± 8.65 ^b
FB3	25	1.20±0.23***	61.93 ± 1.48 ^b	2.40± 0.58*	47.52 ± 1.86 ^b
	50	1.28±0.32***	59.49 ± 3.96 ^b	2.60± 0.71	43.36 ± 3.84 ^b
	100	1.47±0.48***	53.80±8.08 ^b	2.45± 0.71*	46.71 ± 4.53 ^b
FB4	25	1.26±0.36***	60.08 ± 5.24 ^b	2.93± 0.93	36.48 ± 7.18 ^a
	50	1.38±0.16***	55.64 ± 1.71 ^b	2.87± 0.28	36.37 ± 6.92 ^a
	100	1.45±0.12***	53.49±3.39 ^b	2.43± 0.10*	45.70 ± 9.00 ^b
FB5	25	1.12±0.22***	64.26 ± 1.41 ^b	2.66± 0.25	40.87 ± 6.64 ^b
	50	1.17±0.16***	62.45 ± 0.84 ^b	2.91± 0.17	35.11 ± 9.65 ^a
	100	1.00±0.11***	67.94±1.37 ^b	2.63± 0.24	41.50 ± 6.94 ^b

Results are expressed as mean of induction ratio (IR) ± SD (n = 3) with significant difference for *p < 0.05, **p < 0.01, *** p < 0.001 (Dunnett's test) calculated comparing IR values obtained from extracts co-treated with standard genotoxins to IR values obtained from single standard genotoxins. Antigenotoxicity (% mean ± SD) was calculated as follows: $[1 - (\beta\text{galactosidase unit}_{\text{GENOTOXIN+SAMPLE}} / \beta\text{galactosidase unit}_{\text{GENOTOXIN}})] * 100\%$, [31].

NC negative control; 4-NQO 4-nitroquinoline; 2AA 2-amino-anthracene

FL *F.angustifolia* Vahl. leaves; FB *F.angustifolia* Vahl. stem bark

1 Ethanolic; 2 Organic/Ethyl Acetat; 3 Aqueous/ Ethyl Acetat; 4 Organic/ Chloroform; 5 Aqueous/ Chloroform

^aNeutral effect (< 40% Antigenotoxicity)

^bModerate effect (40%– 70% Antigenotoxicity)

^cStrong effect (>70% Antigenotoxicity) [32].

<https://doi.org/10.1371/journal.pone.0230690.t003>

Table 4. MTT assay. IC₅₀ values (µg/mL), with 95% confidence range (in brackets) obtained by MTT assay after 72h treatment of Hep-G2 and MCF7 cell lines with leaves and stem bark extracts of *F.angustifolia* Vahl.

Treatment	Hep-G2		MCF-7	
	IC ₅₀ (confidence range)	LOAEC	IC ₅₀ (confidence range)	LOAEC
FL1	713 (488–1041)	500	496 (281–871)	250
FL2	527 (326–853)	500	1047 (884–1240)	750
FL3	1644 (1453–1862)	1000	1053 (824–1344)	1000
FL4	680 (372–1241)	500	970 (732–1287)	500
FL5	441 (242–801)	125	479 (352–651)	250
FB1	530 (303–929)	500	682 (469–942)	500
FB2	626 (498–787)	500	886 (645–1218)	750
FB3	431 (286–649)	250	882 (627–1240)	750
FB4	777 (664–908)	500	634 (631–932)	100
FB5	504 (411–617)	250	437 (330–578)	125

LOAEC: Lowest Observable Adverse Effect Concentration (µg/mL), (*Dunnett's test*, $p < 0.05$)

FL *F.angustifolia* Vahl. leaves; FB *F.angustifolia* Vahl. stem bark

1 Ethanolic; 2 Organic/Ethyl Acetat; 3 Aqueous/ Ethyl Acetat; 4 Organic/ Chloroform; 5 Aqueous/ Chloroform

<https://doi.org/10.1371/journal.pone.0230690.t004>

are reported in Table 4. Almost completely overlapped 95% confidence intervals of IC₅₀ values were found in all extracts. In addition, the lowest observable adverse effect concentrations (LOAECs, $p < 0.05$) were observed with FL5 (125 µg/mL), FB3 and FB5 (250 µg/mL) on Hep-G2, while FB4 (100 µg/mL), FB5 (125 µg/mL), FL1 and FL5 (250 µg/mL) were obtained on MCF-7. Furthermore, IC₅₀ values of the antiproliferative activity exerted by the extracts on normal-like cells (TelCOFS02MA) were used to calculate SI [34]. Selective toxicity towards cancer cells was shown by FL1 (SI: 2.04, Hep-G2; 2.93, MCF-7), FL5 (SI: 2.40, Hep-G2; 2.21, MCF-7) and FB5 (SI: 2.52, Hep-G2; 2.90, MCF-7).

Phytochemical investigation

Solvent extraction yield and quantification of total phenolics and flavonoids. Extraction yield from raw plant material, total phenolics and flavonoids are reported in Table 5. Ethanolic extracts FL1 and FB1 exhibited a high percentage, 8.5 and 5.9%, respectively. While, the aqueous/chloroform extract FL5 and FB5 showed the lowest percentages (0.7; 0.5%) among all other extracts.

All extracts exhibited the presence of various quantities of total phenolics and flavonoids contents. Leaves extracts contained approximately the same amount of total phenols as the corresponding stem bark extract, while the most prominent being that of the aqueous chloroform fractions (FB5) with 207 µg catechin equivalent/ mg dry extract (Cat. Eq. mg⁻¹ extract). On the other hand, no differences were noticed for flavonoids contents. In fact, the amount ranged from 15.63 to 16.67 µg Rutin Eq mg⁻¹ extract for leaves, and from 10.61 to 17.56 µg Rutin Eq mg⁻¹ extract for stem bark.

UHPLC/MS investigation. The UHPLC/MS analysis was carried out in negative mode, phenolic compounds were identified by examination of retention times, UV -visible and mass spectra, corresponding to the different metabolites eluted from leaves and stem bark extracts of *F. angustifolia* Vahl. the UHPLC chromatograms of the aqueous extract of chloroform (FL5) and its native ethanolic extract (FL1) are presented in Fig 1, the molecular weights and retention times collected from the analysis together with the identifications of the detected metabolites are shown in Table 6.

Table 5. Extraction yield and quantitative phytochemicals screening. Extraction yield and quantitative phytochemicals screening of extracts from *F. angustifolia* Vahl.

<i>F. angustifolia</i> Vahl.	Extraction Yield (g dry extract/100g of powder)	Extraction content	
		Polyphenols ($\mu\text{g Cat. Eq. /mg dry extract}$)	Flavonoids ($\mu\text{g Rut. Eq. /mg dry extract}$)
FL1	8.53	98.63 \pm 6.25	16.22 \pm 5.02
FL2	3.88	105.95 \pm 1.45 ^{ns}	16.67 \pm 1.20 ^{ns}
FL3	5.56	44.94 \pm 4.38 ^{***}	15.90 \pm 2.62 ^{ns}
FL4	5.2	120.65 \pm 2.83 ^{***}	16.67 \pm 1.20 ^{ns}
FL5	0.74	99.52 \pm 1.65 ^{ns}	15.63 \pm 2.91 ^{ns}
FB1	5.85	92.86 \pm 2.91	11.68 \pm 0.49
FB2	1.32	76.13 \pm 0.94 ^{###}	15.93 \pm 5.09 ^{ns}
FB3	4.25	75.30 \pm 1.23 ^{###}	10.61 \pm 0.96 ^{ns}
FB4	3.18	32.56 \pm 0.94 ^{###}	17.56 \pm 3.55 ^{ns}
FB5	0.48	207.08 \pm 4.83 ^{###}	14.25 \pm 3.02 ^{ns}

Data are presented as means \pm SD (n = 3). $\mu\text{g Cat. Eq.}$: microgramme catechin equivalent, $\mu\text{g Rut. Eq.}$: microgramme rutin equivalent.

^{ns} Not significant

^{***} p < 0.001 compared to FL1

^{###} p < 0.001 compared to FSB1. *,# Significance (p < 0.001) by One way ANOVA test, with Dunnett's post test of GraphPad Prism Software. FL *F. angustifolia* Vahl. leaves; FB *F. angustifolia* Vahl. stem bark; 1 Ethanolic; 2 Organic/Ethyl Acetat; 3 Aqueous/ Ethyl Acetat; 4 Organic/Chloroform; 5 Aqueous/ Chloroform

<https://doi.org/10.1371/journal.pone.0230690.t005>

The shared metabolites between these two extracts, with more intensities in the aqueous phase (in bold, Table 6) are in the first place, oleuropein (4), a major compound, common to Oleaceae family (especially *Fraxinus* species) derived from the biosynthetic pathway of a secoiridoid [37], its main metabolites are tyrosol and hydroxytyrosol [38]. Two other metabolites belonging to this same chemical group, ligstroside (6) with an intensity twice as high in the

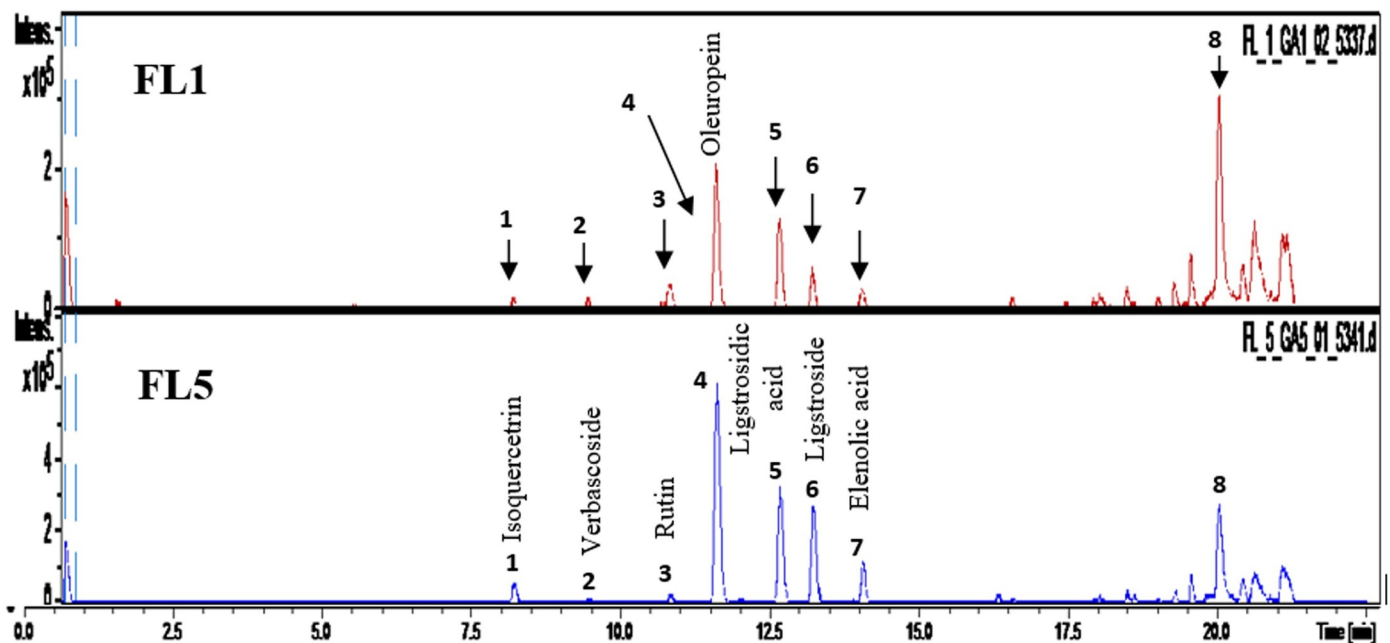


Fig 1. Leaves HPLC chromatogram. HPLC chromatogram (254 nm) of the composition of the aqueous chloroform (FL5) extract and its native crude extract (FL1) of *F. angustifolia* Vahl leaves. FL1: Ethanolic extract; FL5: aqueous/ chloroform extract.

<https://doi.org/10.1371/journal.pone.0230690.g001>

Table 6. Retention times (t_r) of the ions (m/z) and the molecular weights. Table of the retention times (t_r) of the ions (m/z) and the molecular weights corresponding to the peaks revealed in the HPLC/MS spectra of *F. angustifolia* Vahl. leaves extracts.

Extract	N°	t_r (min)	m/z Experimental	Max MW	Intensity	Molecular formula	Identified metabolite
FL1	1	1.0	181.10	182.11	20816	C ₉ H ₁₀ O ₄	Syringaldehyd
	2	7.9	463.19	464.20	28659	C ₂₁ H ₂₀ O ₁₂	Isoquercitrin
	3	10.5	609.20	610.21	52629	C ₂₇ H ₃₀ O ₁₆	Rutin
	4	11.4	539.23	540.23	236032	C₂₅H₃₂O₁₃	Oleuropein
	5	12.5	569.24	570.25	266002	---	Ligstrosidic acid
	6	13.1	523.24	524.24	98953	C ₂₅ H ₃₂ O ₁₂	Ligstroside
	7	14.0	601.27	602.27	43358	---	Derivative of Elenolic acid
	8	16.5	909.38	910.39	28220	---	Isomer of GL5
	9	20.3	698.97	699.98	80336	---	Gallic acid dihexoside sinapoyl
	10	20.4	532.97	533.98	117390	C₂₄H₂₂O₁₄	Kaempferol-3-O-malonyl glucoside
	11	21.0	255.26	256.27	27040	C ₁₅ H ₁₂ O ₄	Liquirtigenin
FL5	1	7.9	463.19	464.19	30623	C ₂₁ H ₂₀ O ₁₂	Isoquercitrin
	2	9.2	623.25	624.26	12982	C ₂₉ H ₃₆ O ₁₅	Verbascoside /Isoverbascoside
	3	10.5	609.20	610.21	16134	C ₂₇ H ₃₀ O ₁₆	Rutin
	4	11.4	539.22	540.23	392912	C₂₅H₃₂O₁₃	Oleuropein
	5	12.5	569.24	570.24	276806	C₂₆H₃₄O₁₄	Ligstrosidic acid
	6	13.1	523.23	524.24	208500	C₂₅H₃₂O₁₂	Ligstroside
	7	14.0	601.26	602.27	69027	---	Derivative of Elenolic acid

FL: *F. angustifolia* Vahl. leaves; 1: Ethanolic extract; 5: Aqueous/chloroform extract

In Bold: identified metabolite with high intensity

<https://doi.org/10.1371/journal.pone.0230690.t006>

aqueous extract as the ethanolic extract and the compound (5) supposed to be ligstrosidic acid, according to the bibliographic data corresponding to the results of the UHPLC / MS analysis of the fraction where this metabolite is the major one. It is also remarkable that these two extracts share two flavonols glycosides, isoquercitrin (1) and rutin (3), they are glycoside derivatives of quercetin, which were previously reported as the constituents of genus *Fraxinus* has been reported [39]. There is also a compound whose intensity is not very different, elenolic acid (7), which would come from the hydrolysis of oleuropein which releases hydroxytyrosol and elenolic acid. Tyrosol, on the other hand, comes from the degradation of ligstroside [40]. Finally, we note the presence of a low intensity peak of a phenylethanoid, verbascoside (2), previously reported in several *Fraxinus* species.

HPLC chromatograms and identified metabolites in the aqueous extracts of chloroform (FB5) and its native ethanolic extract (FB1) are illustrated in Fig 2 and Table 7.

It is noted in this comparative figure that FB5 concentrated and selected mainly phenylethanoids and secoiridoids compared to the more diverse composition in metabolites with lower incidences in the native extract (FB1). The two major compounds of this extract are calceolarioside B and ligstroside. Their isomers and derivatives, namely at least two isomers of calceolarioside B, the verbascoside and its isoverbascoside isomer, and its decaffeoyl derivative. Similarly, lower levels were found for ligstroside, demethyl-ligstroside and ligstrosidic acid. This extract also selected a small amount of a coumarin common in the genus *Fraxinus*, fraxin.

Discussion

Too often, treatments based on medicinal plants can cause serious health risks [41]. Thus, it was consistent to assess the mutagenic and genotoxic activities of *F. angustifolia* Vahl. extracts in order to exclude their eventual negative impact on genetic material.

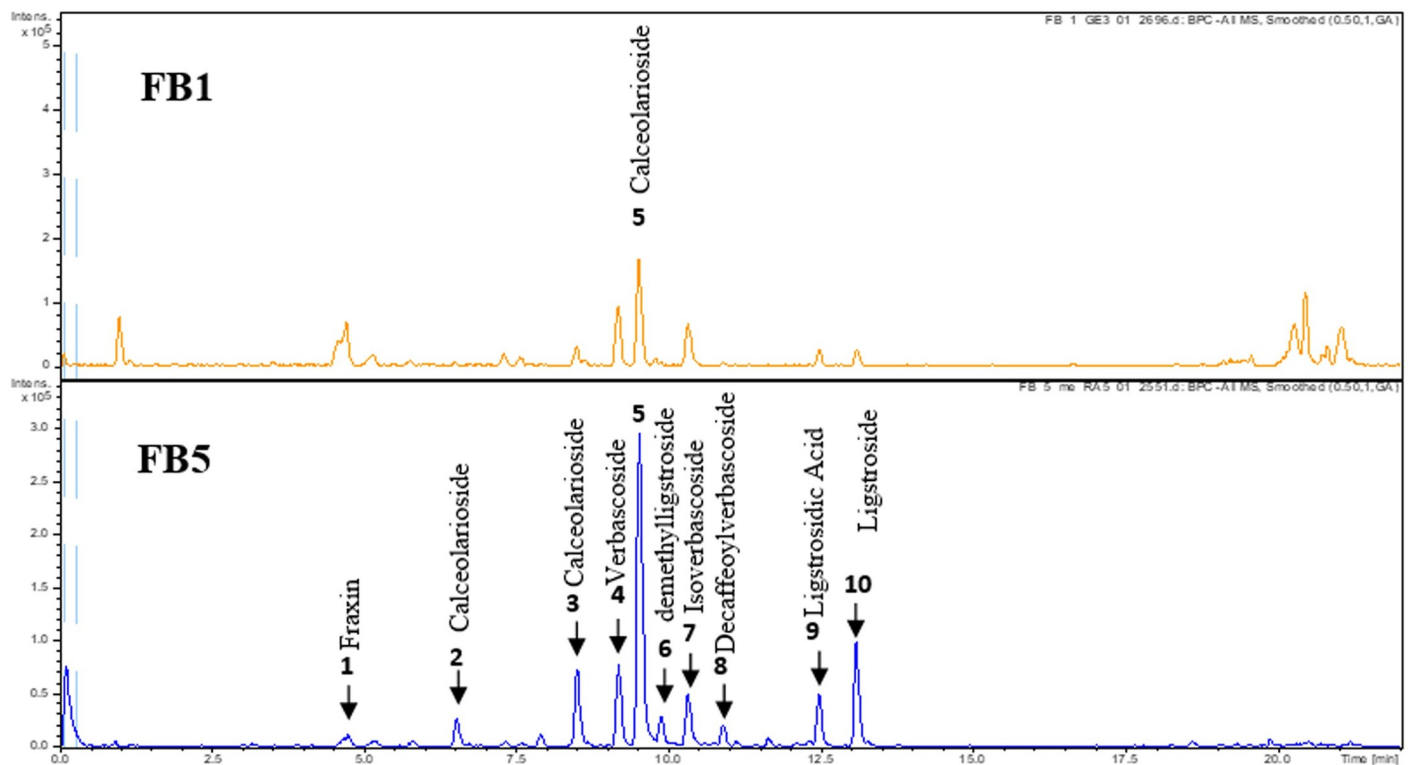


Fig 2. Bark HPLC chromatogram. HPLC chromatogram (254 nm) of the composition of the aqueous chloroform (FSB5) extract and its native crude extract (FB1) of *F. angustifolia* Vahl stem bark. FB1 ethanolic extract; FB5 aqueous/ chloroform extract.

<https://doi.org/10.1371/journal.pone.0230690.g002>

In this initial report we demonstrated that the tested *F. angustifolia* Vahl. extracts did not produce DNA lesions. The absence of genotoxicity/mutagenicity is not typical of all natural products in use, since several medicinal plants, assayed with the *umu* and/or the Ames test, in the presence or absence of the S9 metabolic activation, proved to be positive for genotoxicity/mutagenicity [32, 42–44]. Polyphenols are a diverse class of compounds; many are favorable in preventing disease and protecting the stability of the genome. However not all polyphenols and not all their actions are necessarily advantageous. Some of them have mutagenic and/or pro-oxidant effects. It was proven that several polyphenols, including quercetin, can bind to DNA and this direct interaction may be an important mechanism of bacterial mutagenicity, even those polyphenols that are negative in bacterial systems may be clastogenic in mammalian cells [45].

Furthermore, conflicting results about the mutagenic potential of verbascoside, a phenylethanoid compound, identified in *F. angustifolia* Vahl. when tested on TA 98 and TA 100, did not induce frameshift and base-pair substitution mutations in the presence or absence of metabolic system [46], but induced DNA damages on human lymphocytes, with an involvement of PARP-1 and p53 expression through its caffeic acid moiety [47].

Recently, *in vivo* subsequent tests on *Drosophila melanogaster* and rabbit reported that verbascoside do not give rise to any mutagenic activity at any of the tested concentrations [48–50]. Besides poor *in vivo* bioavailability of verbascoside was reported and could be also related to this behavior [51].

Substances with antigenotoxic **and/or** antimutagenic properties may be useful against the damage caused by environmental mutagens. The antigenotoxic study of *F. angustifolia* Vahl.

Table 7. Retention times (t_r) of the ions (m/z) and the molecular weights. Table of the retention times (t_r) of the ions (m/z) and the molecular weights corresponding to the peaks revealed in the HPLC/MS spectra of *F. angustifolia* Vahl. stem bark extracts.

Extract	N°	t_R (min)	m/z Experimental	Max MW	Intensity	Molecular formula	Identified metabolite
FB 1	1	1.0	341.15	342.15	81408	C ₁₅ H ₁₈ O ₉	Caffeic acid glucoside
	2	4.7	369.13	370.13	68630	C ₁₆ H ₁₈ O ₁₀	Fraxin
	3	5.1	429.15	430.15	20964	---	---
	4	7.3	535.23	536.24	21362	C ₂₆ H ₃₂ O ₁₂	8-Hydroxypinoresinol-4/8-glucoside.
	5	8.5	477.18	478.19	30486	C ₂₃ H ₂₅ O ₁₁	Calceolarioside A
	6	9.2	623.25	624.26	95389	C ₂₉ H ₃₆ O ₁₅	Verbascoside
	7	9.5	477.19	478.19	174206	C₂₃H₂₅O₁₁	Calceolarioside B
	8	10.3	623.25	624.26	67733	C ₂₉ H ₃₆ O ₁₅	Isoverbascoside
	9	12.5	569.24	570.25	26693	C ₂₆ H ₃₄ O ₁₄	Ligstrosidic acid
	10	13.1	523.23	524.24	28317	C ₂₅ H ₃₂ O ₁₂	Ligstroside
	11	19.6	269.22	270.23	21594	C ₈ H ₁₃ O ₁₀	Apigenin
	12	20.3	698.97	699.98	68940	---	Gallic acid dihexosidesinapoyl
	13	20.5	532.98	533.98	120273	C₂₄H₂₂O₁₄	Kaempferol-3-O-malonyl glucoside
	14	21.0	255.26	256.27	62872	C ₁₅ H ₁₂ O ₄	Liquirtigenin
FB 5	1	4.7	369.12	370.13	10714	C ₁₆ H ₁₈ O ₁₀	Fraxin
	2	6.5	477.18	478.19	27245	C ₂₃ H ₂₅ O ₁₁	Calceolarioside
	3	7.9	477.18	478.19	12107	C ₂₃ H ₂₅ O ₁₁	Calceolarioside
	4	9.2	623.25	624.26	81456	C ₂₉ H ₃₆ O ₁₅	Verbascoside
	5	9.5	477.18	478.19	299210	C₂₃H₂₅O₁₁	Calceolarioside B
	6	9.9	509.21	510.22	27567	C ₂₄ H ₃₀ O ₁₂	demethyligstroside
	7	10.3	623.25	624.26	50994	C ₂₉ H ₃₆ O ₁₅	Isoverbascoside
	8	10.9	461.19	462.19	19841	C ₂₀ H ₂₉ O ₁₂	Decaffeoylverbascoside
	9	12.5	569.24	570.24	51995	C ₂₆ H ₃₄ O ₁₄	Ligstrosidic acid
	10	13.1	523.23	524.23	102076	C₂₅H₃₂O₁₂	Ligstroside

FB: *F. angustifolia* Vahl. stem bark; I: Ethanolic extract; 5: Aqueous/chloroform extract

In Bold: identified metabolite with high intensity

<https://doi.org/10.1371/journal.pone.0230690.t007>

extracts was first evaluated by the *umu* test towards the direct mutagen 4-NQO, revealing to have a strong antigenotoxic effect (FL5 and FB5 extracts, inhibition by 70%) besides a moderate effect observed with the indirect mutagen 2-AA. This indicates that these extracts may not be involved in 2-AA genotoxic action but in the 4-NQO mutagenic mechanism.

4-NQO induces intracellular oxidative stress; it can undergo redox cycling and generate reactive oxygen species such as superoxide radical and hydrogen peroxide [52, 53]. Furthermore, *F. angustifolia* Vahl. has revealed a potent antioxidative capacity [19, 26]; Hence, the possible mechanism of the observed antigenotoxic potency could involve the bioactive phenolic antioxidant compounds present in the *F. angustifolia* Vahl. extracts which might interact with the reactive intermediates of 4-NQO.

The second known mechanism of the carcinogenic action of 4-NQO is the formation of stable mono-adducts on purines [54, 55]. This damage could be repaired by the nucleotide excision repair pathway [54]. Moreover, the strain TA1553 /pSK 1002 is *uvrB* deficient [56]. Taken altogether, these results indicate that, in addition to antioxidant activity *F. angustifolia* Vahl., leaves and stem bark extracts may exert their antimutagenic properties also by modulating the DNA repair processes [57].

Umu test showed lower detection sensitivities to the weaker Ames positive chemicals of relatively strong Ames positive [58]. Moreover, Oda in 2016 [59] concluded that *umu* and Ames

test are complementary and would be used together in order to broaden the detection capacity. In fact, *umu* test detects damage to DNA by evaluating the expression level of the *umu* gene product, which is one of the DNA repairing enzymes induced through the SOS response while Ames assay detects the mutation of gene for histidine synthesis by growing a mutant colony that is a phenotype of such mutation [58].

In the present study, a variation of Ames test was used to assess the antimutagenic activity of *F.angustifolia* Vahl. extracts against other mutagens (2-NF, NaN₃, 3-MC and CP) with different mutagenic mechanism than that used for *umu* test. In addition, extracts of *F. angustifolia* Vahl. were subjected to two concentrations of the standard mutagens for better understanding of the dose effect relation.

F. angustifolia Vahl. leaves and stem bark extracts exhibited a protective effect against low concentrations of mutagens, on both strains TA98 and TA100 independently tested with or without metabolic activation. Regarding high concentrations of standard mutagen, the extracts were effective on TA100 and less active on TA98, when tested in the absence of metabolic activation. The extracts acted by interrupting several mutagenesis processes, indicating that they may directly protect the DNA from damages, possibly by quick elimination of mutagens from the bacteria before their interaction with the DNA. They may also facilitate or stimulate the bacterial transmembrane export system to eliminate the mutagens, or interfere with the uptake of mutagens into bacteria [57, 60]. However, the inhibition of mutagenesis is often complex, involving multiple mechanisms [61].

Otherwise, cytotoxicity and selectivity of *F. angustifolia* Vahl. were examined on normal-like cells (human fibroblasts, TelCOFS02MA), human hepatocarcinoma cells (HepG2) and human adenocarcinoma cells (MCF-7). Overall, it is interesting to observe a correlation between phytochemicals profile identified by HPLC/MS and the selectivity degree of the extracts. FL1 and FL5 were selective towards the two cancer cell lines, and this may be due to the high content of oleuropein and ligstrosidic acid detected in the two extracts; oleuropein is a characteristic phenol largely distributed in Oleaceae family, and involved in a number of exhaustively documented biological activities [62]. Oleuropein exhibited cytotoxic activity against six tumoral cell lines, MCF-7, HepG2, Caco-2, A549, MDA, and DU145 [63]. Furthermore, Goldsmith and collaborators [64] assessed the anti-pancreatic cancer potential of oleuropein and its metabolite hydroxytyrosol on pancreatic cancer cells **and non-tumorigenic** pancreas cells inducing apoptosis in tumor cells, and displaying a protective effect **on non-tumorigenic** cells. The authors argued that this selectivity to the sensitivity of cancer cells is due to ROS generation. In fact, many human cancer cell types exist in a highly oxidative state compared to their normal tissues and, therefore, the selective activity of oleuropein on tumor cells could be due to their increased sensitivity towards ROS [64].

Moreover, it has been previously shown that oleuropein inhibits cell proliferation on MCF7 and HepG2 by decreasing the gene expression involved in metastasis and invasion of tumoral cells [65, 66]. Concerning ligstrosidic acid no data are available on biological activities and therefore no conclusion can be drawn.

In stem bark extracts, only FB5 showed significant SI values with 2.52 for HepG2 and 2.90 for MCF-7 while no selectivity was observed in the native crude extract FB1. Indeed, FB5 has the highest amount of polyphenol among all extract, moreover a net difference in the intensity peak of ligstroside and calceolarioside B in FB5 suggests that it could be involved in its antiproliferative activity. There is no extensive data about studies testing the biological activity of ligstroside and calceolarioside B. Principally, ligstroside aglycon was checked about possible implication against two proteins involved in breast cancer, with significant results even at low doses [67, 68].

However, it cannot be excluded that some minor components identified in the different extracts such as rutin, isoquercitrin, verbascoside and its isomer isoverbascoside may also

exert pharmacological effects and therefore play a crucial role in the proprieties exerted in this study [68, 69].

Conclusion

In conclusion, the results of the present study showed that *F. angustifolia* Vahl. leaves and stem bark extracts alone had no mutagenic effect on the tested strains, either in the presence or absence of metabolic activation. Moreover, significant antimutagenic activity of these extracts against several mutagens, indicates that *F. angustifolia* Vahl. leaves and stem bark extracts may directly protect DNA damage from mutagens, these *in vitro* test results would raise the question of its relation to efficacy under *in vivo* conditions in which factors such as bioavailability and metabolism must be taken into account.

More studies need to be carried out to assess the antimutagenic mechanisms of isolated phytochemical components of leaves and stem bark extracts of *F. angustifolia* Vahl. In addition to this, the antimutagenic activity of these extracts should be tested on *in vivo* model.

The strong cytotoxic activity and selectivity of FL1, FL5 and FB5 extracts against HepG2 and MCF-7 could be an indication on the potentiality of these extracts to be further screened for antiproliferative or cytotoxic activities against a number of cancer cell lines. It could be further studied for inhibitory effects on the invasion of cells at a non-lethal concentration.

Supporting information

S1 Fig. Direct mutagenic activity. Mutagenic activity of *F. angustifolia* Vahl. extracts (1000 µg/mL) in Ames test in the absence of the exogenous metabolic activation system (S9). (DOCX)

S2 Fig. Indirect mutagenic activity. Indirect mutagenic activity of *F. angustifolia* Vahl. extracts (1000 µg/mL) in Ames test in the presence of the exogenous metabolic activation system (S9). (DOCX)

S1 Table. Genotoxicity. Genotoxicity of different extracts of *F. angustifolia* Vahl. (1000 µg/mL) in the absence and in presence of the exogenous metabolic activation system (S9). (DOCX)

Author Contributions

Conceptualization: Ghania Bouguellid, Rob Verpoorte, Marina Isidori.

Data curation: Chiara Russo, Karima Ayouni, Erica Wilson.

Investigation: Ghania Bouguellid, Chiara Russo, Margherita Lavorgna, Concetta Piscitelli, Karima Ayouni, Erica Wilson, Hye Kyonn Kim, Rob Verpoorte, Young Hae Choi, Dina Kilani-Atmani, Djebbar Atmani, Marina Isidori.

Methodology: Ghania Bouguellid, Margherita Lavorgna, Concetta Piscitelli, Karima Ayouni, Erica Wilson, Hye Kyonn Kim, Young Hae Choi, Dina Kilani-Atmani.

Supervision: Karima Ayouni, Rob Verpoorte, Djebbar Atmani, Marina Isidori.

Visualization: Margherita Lavorgna, Concetta Piscitelli, Dina Kilani-Atmani.

Writing – original draft: Ghania Bouguellid, Chiara Russo, Marina Isidori.

Writing – review & editing: Rob Verpoorte, Djebbar Atmani, Marina Isidori.

References

1. Atanasov AG, Waltenberger B, Pferschy-Wenzig EM, Linder T, Wawrosch C, Uhrin P, et al. Discovery and resupply of pharmacologically active plant-derived natural products: A review. *Biotechnology Advances*. 2015; 33(8): 1582–1614. <https://doi.org/10.1016/j.biotechadv.2015.08.001> PMID: 26281720
2. Lunenfeld B, Stratton P. The clinical consequences of an ageing world and preventive strategies. *Best Practice & Research: Clinical Obstetrics & Gynaecology*. 2013; 27(5): 643–59.
3. Torgovnick A, Schumacher B. DNA repair mechanisms in cancer development and therapy. *Frontiers in Genetics*. 2015; 23(6):157.
4. Nagarathna PKM, Johnson Wesley M, Sriram Reddy P, Reena K. Review on Genotoxicity, its Molecular Mechanisms and Prevention. *International Journal of Pharmaceutical Sciences Review and Research*. 2013; 22(1): 236–243.
5. Stoczyńska K, Powroźnik B, Pękala E, Waszkielewicz AM. Antimutagenic compounds and their possible mechanisms of action. *Journal of Applied Genetics*. 2014; 55: 273–285 <https://doi.org/10.1007/s13353-014-0198-9> PMID: 24615570
6. Collins AR, Azqueta A, Langie SAS. Effects of micronutrients on DNA repair. *European Journal of Nutrition*. 2012; 51: 261–279. <https://doi.org/10.1007/s00394-012-0318-4> PMID: 22362552
7. Orhan F, Gulluce M, Ozkan H, Alpsoy L. Determination of the antigenotoxic potencies of some luteolin derivatives by using a eukaryotic cell system, *Saccharomyces cerevisiae*. *Food Chemistry*. 2013; 141: 366–372. <https://doi.org/10.1016/j.foodchem.2013.02.089> PMID: 23768369
8. Chatterjee S, Poduval TB, Tilak JC, Devasagayam TPA. A modified, economic, sensitive method for measuring total antioxidant capacities of human plasma and natural compounds using Indian saffron (*Crocus sativus*). *Clinica Chimica Acta*. 2005; 352: 155–163.
9. Nsimba RY, Kikuzaki H, Konishi Y. Antioxidant activity of various extracts and fractions of *Chenopodium quinoa* and *Amaranthus* spp. seeds. *Food Chemistry*. 2008; 106: 760–766.
10. Beloued A. *Plantes Médicinales d'Algérie*. Office des publications universitaires. Alger. 1998.
11. Baba-Aissa F. *Encyclopédie des Plantes Utiles. Flore d'Algérie et du Maghreb, Substances Végétales d'Afrique d'Orient et d'Occident*. EDAS, Librairie Moderne, Rouiba. 1999.
12. Duke JA, Duke PAK. *Duke's handbook of medicinal plants of the Bible*. Boca Raton, Florida. 2008.
13. Macaluso AM. Il frassino da manna in Sicilia (*Fraxinus ornus*—*Fraxinus excelsior*). F. Battiato ed., Catania. 1921.
14. Oddo E, Sajeva M, Bellini E. Seasonal pattern of mannitol and malate accumulation in leaves of two manna ash species (*Fraxinus ornus* L. and *F. angustifolia* Vahl) growing in Sicily. *Plant Biosystems—An International Journal Dealing with All Aspects of Plant Biology*. 2002; 136: 29–34.
15. Schicchi R, Camarda L, Spadaro V, Pitonzo R. Caratterizzazione chimica della manna estratta nelle Madonie (Sicilia) da cultivar di *Fraxinus angustifolia* e di *Fraxinus ornus* (Oleaceae). *Quaderni di Botanica Ambientale e Applicata* 17/2. 2006. pp. 139–162.
16. Lazzarini E, Lonardoni AR. *La manna salute della natura*. Edizioni Mediterranee, 1984.
17. Camarda L, Di Stefano V, Pitonzo R. Natural resins: chemical constituents and medicinal uses. 2011. Available from: <https://www.researchgate.net/publication/287289098>
18. EMA (European medicine agency) Assessment report on *Fraxinus excelsior* L. or *Fraxinus angustifolia* Vahl., folium. Committee on Herbal Medicinal Products (HMPC). 2012; 239270: 5–7.
19. Ayouni K, Berboucha-Rahmani M, Kim HK, Atmani D, Verpoorte R, Choi YH. Metabolomic tool to identify antioxidant compounds of *Fraxinus angustifolia* leaf and stem bark extracts. *Industrial Crops and Products*. 2016; 88: 65–77.
20. Berboucha M, Ayouni K, Atmani D, Atmani D, Benboubetra M. Kinetic Study on the Inhibition of Xanthine Oxidase by Extracts from Two Selected Algerian Plants Traditionally Used for the Treatment of Inflammatory Diseases. *Journal of Medicinal Food*. 2010; 13: 896–904. <https://doi.org/10.1089/jmf.2009.0164> PMID: 20553185
21. Falé PL, Ferreira C, Rodrigues AM, Cleto P, Madeira PJA, Florêncio MH, et al. Antioxidant and anti-acetylcholinesterase activity of commercially available medicinal infusions after in vitro gastrointestinal digestion. *Journal of Medicinal Plants Research*. 2013; 7: 1370–1378.
22. Ahmane N, Atmani-Kilani D, Chafer N, Ayouni K, Rahmani-Berboucha M, Costa GD, et al. Identification of bioactive compounds from *Fraxinus angustifolia* extracts with anti-NADH oxidase activity of bovine milk xanthine oxidoreductase. *Turkish Journal of Biology*. 2019; 43: 133–147. <https://doi.org/10.3906/biy-1810-26> PMID: 31410081
23. Medjahed Z., Atmani-Kilani D., Fauconnier M.-L., Richard G., and Atmani D. Hepatoprotective and anti-diabetic activities of *Fraxinus angustifolia* Vahl extracts in animal models: characterization by high

- performance liquid chromatography analysis. Turkish Journal of Medical Sciences. 2016; 46, 910–920. <https://doi.org/10.3906/sag-1502-13> PMID: 27513273
24. Moulaoui K, Caddeo C, Manca ML, Castangia I, Valenti D, Escribano E, et al. Identification and nano-trapment of polyphenolic phytocomplex from *Fraxinus angustifolia*: In vitro and in vivo wound healing potential. European Journal of Medicinal Chemistry. 2015; 89: 179–188. <https://doi.org/10.1016/j.ejmech.2014.10.047> PMID: 25462238
 25. Chiang YL, Chan EWL, Tan PL, Lim YY, Stanslas J, Goh JK. Xanthine oxidase inhibitors from the leaves of *Alsophila spinulosa* (Hook) Tryon. Journal of Enzyme Inhibition. 1994; 8(1): 61–71. <https://doi.org/10.3109/14756369409040777> PMID: 7539070
 26. Atmani D, Chaheer N, Berboucha M, Ayouni K, Lounis H, Boudaoud H, et al. Antioxidant capacity and phenol content of selected Algerian medicinal plants. Food Chemistry. 2009; 112: 303–309.
 27. Maron DM, Ames BN. Revised methods for the Salmonella mutagenicity test. Mutation Research. 1983; 113(3–4):173–215. [https://doi.org/10.1016/0165-1161\(83\)90010-9](https://doi.org/10.1016/0165-1161(83)90010-9) PMID: 6341825
 28. Mortelmans K, Zeiger E. The Ames Salmonella/microsome mutagenicity assay. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 2000; 455: 29–60. [https://doi.org/10.1016/s0027-5107\(00\)00064-6](https://doi.org/10.1016/s0027-5107(00)00064-6) PMID: 11113466
 29. Resende FA, Campos DL, da Silva VC, De Grandis RA, Souza LP, Leonardo Junior CS, et al. Mutagenicity and chemopreventive activities of Astronium species assessed by Ames test. Regulatory Toxicology and Pharmacology. 2015; 72: 506–513. <https://doi.org/10.1016/j.yrtph.2015.05.014> PMID: 26002624
 30. ISO/DIS 13829. Water Quality Determination of the Genotoxicity of Water and Waste Water Using the Umu-Test. 2000.
 31. Skrzypczak A, Przystupa N, Zgadzaj A, Parzonko A, Sykłowska-Baranek K, Paradowska K, et al. Antigenotoxic, anti-photogenotoxic and antioxidant activities of natural naphthoquinone shikonin and acetylshikonin and *Arnebia euchroma* callus extracts evaluated by the umu-test and EPR method. Toxicology in Vitro. 2015; 30: 364–372. <https://doi.org/10.1016/j.tiv.2015.09.029> PMID: 26434532
 32. Caillet S, Lessard S, Lamoureux G, Lacroix M. Umu test applied for screening natural antimutagenic agents. Food Chemistry. 2011; 124: 1699–1707.
 33. Baharum Z, Akim AM, Taufiq-Yap YH, Hamid RA, Kasran R. In vitro antioxidant and antiproliferative activities of methanolic plant part extracts of *Theobroma cacao*. Molecules. 2014; 19(11): 18317–18331. <https://doi.org/10.3390/molecules191118317> PMID: 25389662
 34. Demirgan R, Karagöz A, Pekmez M, Önay-Uçar E, Artun FT, Gürer Ç, et al. In vitro anticancer activity and cytotoxicity of some papaver alkaloids on cancer and normal cell lines. African Journal of Traditional, Complementary and Alternative Medicines. 2016; 13: 22.
 35. Kähkönen MP, Hopia AI, Vuorela HJ, Rauha JP, Pihlaja K, Kujala TS, et al. Antioxidant activity of plant extracts containing phenolic compounds. Journal of Agriculture and Food Chemistry. 1999; 47: 3954–3962.
 36. Maksimovic Z, Malencic Đ, Kovacevic N. Polyphenol contents and antioxidant activity of maydis stigma extracts. Bioresources Technology. 2005; 96: 873–877.
 37. Visioli F, Bellomo G, Galli C. Free radical-scavenging properties of olive oil polyphenols. Biochemical and Biophysical Research Communications. 1998; 247: 60–64. <https://doi.org/10.1006/bbrc.1998.8735> PMID: 9636654
 38. Owen RW, Mier W, Giacosa A, Hull WE, Spiegelhalder B, Bartsch H. Identification of lignans as major components in the phenolic fraction of olive oil. Clinical Chemistry. 2000; 46: 976–988. PMID: 10894841
 39. Kostova I, Iossifova T. Chemical components of *Fraxinus* species. Fitoterapia. 2007; 78: 85–106. <https://doi.org/10.1016/j.fitote.2006.08.002> PMID: 17184933
 40. Coxam V, Wauquier F, Darie C, Spilmont M, Davicco MJ, Wittrant Y. Huile d'olive et santé osseuse. 2014; 21(5):7.
 41. Sofowora A, Ogunbodede E, Onayade A. The role and place of medicinal plants in the strategies for disease prevention. African Journal of Traditional, Complementary and Alternative Medicines. 2013; 10(5): 210–229.
 42. Elgorashi EE, Taylor JLS, Maes A, de Kimpe N, van Staden J, Verschaeve L, et al. The use of plants in traditional medicine: potential genotoxic risks. South African Journal of Botany. 2002; 68: 408–410.
 43. De Carvalho MCRD, Barca FNTV, Agnez-Lima LF, De Medeiros SRB. Evaluation of mutagenic activity in an extract of pepper tree stem bark (*Schinus terebinthifolius* Raddi). Environmental and Molecular Mutagenesis. 2003; 42: 185–191. <https://doi.org/10.1002/em.10183> PMID: 14556225

44. Abudayyak M, özdemir Nath E, özZhan G. Toxic potentials of ten herbs commonly used for aphrodisiac effect in Turkey. *Turkish Journal of Medical Sciences*. 2015; 45: 496–506. <https://doi.org/10.3906/sag-1401-153> PMID: 26281312
45. Ferguson LR. Role of plant polyphenols in genomic stability. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*. 2001; 475: 89–111. [https://doi.org/10.1016/s0027-5107\(01\)00073-2](https://doi.org/10.1016/s0027-5107(01)00073-2) PMID: 11295156
46. Henn JG, Steffens L, de Moura Sperotto ND, de Souza Ponce B, Veríssimo RM, Boaretto FBM, et al. Toxicological evaluation of a standardized hydroethanolic extract from leaves of *Plantago australis* and its major compound, verbascoside. *Journal of Ethnopharmacology*. 2019; 229: 145–156. <https://doi.org/10.1016/j.jep.2018.10.003> PMID: 30316886
47. Santoro A, Bianco G, Picerno P, Aquino RP, Autore G, Marzocco S, et al. Verminoside- and verbascoside-induced genotoxicity on human lymphocytes: Involvement of PARP-1 and p53 proteins. *Toxicology Letters*. 2008; 178: 71–76. <https://doi.org/10.1016/j.toxlet.2008.02.006> PMID: 18395372
48. Santos-Cruz L.F., Ávila-Acevedo J.G., Ortega-Capitaine D., Ojeda-Duplancher J.C., Perdígón-Moya J. L., Hernández-Portilla L.B., et al. Verbascoside is not genotoxic in the ST and HB crosses of the *Drosophila* wing spot test, and its constituent, caffeic acid, decreases the spontaneous mutation rate in the ST cross. *Food and Chemical Toxicology*. 2012; 50: 1082–1090. <https://doi.org/10.1016/j.fct.2011.12.006> PMID: 22197714
49. Anter J, Tasset I, Demyda-Peyras S, Ranchal I, Moreno-Millan M, Romero-Jimenez M, et al. Evaluation of potential antigenotoxic, cytotoxic and proapoptotic effects of the olive oil by-product «alperujo», hydroxytyrosol, tyrosol and verbascoside. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. 2014; 772: 25–33. <https://doi.org/10.1016/j.mrgentox.2014.07.002> PMID: 25308544
50. Perucatti A, Genuardo V, Pauciullo A, Iorio C, Incarnato D, Rossetti C, et al. Cytogenetic tests reveal no toxicity in lymphocytes of rabbit (*Oryctolagus cuniculus*, 2n = 44) feed in presence of verbascoside and/or lycopene. *Food and Chemical Toxicology*. 2018; 114: 311–315. <https://doi.org/10.1016/j.fct.2018.02.053> PMID: 29496527
51. Cardinali A, Rotondo F, Minervini F, Linsalata V, D'Antuono I, Debellis L, et al. Assessment of verbascoside absorption in human colonic tissues using the Ussing chamber model. *Food Research International*. 2013; 54: 132–138.
52. Nunoshiba T, Demple B. Potent intracellular oxidative stress exerted by the carcinogen 4-nitroquinoline-N-oxide. *Cancer Research*. 1993; 53: 3250–3252. PMID: 8391920
53. Gallagher JEG, Zheng W, Rong X, Miranda N, Lin Z, Dunn B, et al. Divergence in a master variator generates distinct phenotypes and transcriptional responses. *Genes & Development*. 2014; 28: 409–421.
54. Ikenaga M, Ichzkawa-Ryoand H, Kondo S. The Major Cause of Inactivation and Mutation by 4-Nitroquinoline I-Oxide in *Escherichia coli*: Excisable 4NQO-Purine Adducts. *Journal of Molecular Biology*. 1975; 92: 341–356. [https://doi.org/10.1016/0022-2836\(75\)90233-8](https://doi.org/10.1016/0022-2836(75)90233-8) PMID: 806692
55. Bailleul B, Daubersies P, Galiegue-Zouitina S, Loucheux-Lefebvre MH. Molecular basis of 4-nitroquinoline 1-oxide carcinogenesis. *Japanese Journal of Cancer Research*. 1989; 80: 691–967. <https://doi.org/10.1111/j.1349-7006.1989.tb01698.x> PMID: 2511172
56. Oda Y, Nakamura S, Oki I, Kato T, Shinagawa H. Evaluation of the new system (umu-test) for the detection of environmental mutagens and carcinogens. *Mutation Research/Environmental Mutagenesis and Related Subjects*. 1985; 147: 219–229.
57. Ferguson LR, Philpott M, Karunasinghe N. Dietary cancer and prevention using antimutagens. *Toxicology*. 2004; 198: 147–159. <https://doi.org/10.1016/j.tox.2004.01.035> PMID: 15138038
58. Ota M, Nakamura Y, Kitamoto S, Morimoto T. Alternative Methods for the Safety Evaluation of Chemicals. *Sumitomo Kagaku*. 2005; 2: 1–11.
59. Oda Y. Development and progress for three decades in umu test systems. *Genes and Environment*. 2016; 38(24): 1–14.
60. Resende FA, Munari CC, de Azevedo Bentes Monteiro Neto M, Tavares DC, Bastos JK, da Silva Filho AA, et al. Comparative Studies of the (Anti) Mutagenicity of *Baccharis dracunculifolia* and Artepillin C by the Bacterial Reverse Mutation Test. *Molecules*. 2012; 17: 2335–2350. <https://doi.org/10.3390/molecules17032335> PMID: 22367028
61. Zahin M, Aqil F, Ahmad I. Broad spectrum antimutagenic activity of antioxidant active fraction of *Punica granatum* L. peel extracts. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. 2010; 703: 99–107.
62. Farooqi A, Fayyaz S, Silva A, Sureda A, Nabavi S, Mocan A, et al. Oleuropein and Cancer Chemoprevention: The Link is Hot. *Molecules*. 2017; 22(5): 1–13.

63. Casaburi I, Puoci F, Chimento A, Sirianni R, Ruggiero C, Avena P, et al. Potential of olive oil phenols as chemopreventive and therapeutic agents against cancer: A review of in vitro studies. *Molecular Nutrition & Food Research*. 2013; 57: 71–83.
64. Goldsmith C, Bond D, Jankowski H, Weidenhofer J, Stathopoulos C, Roach P, et al. The Olive Biophenols Oleuropein and Hydroxytyrosol Selectively Reduce Proliferation, Influence the Cell Cycle, and Induce Apoptosis in Pancreatic Cancer Cells. *International Journal of Molecular Sciences*. 2018; 19(7): 1937.
65. Elamin MH, Daghestani MH, Omer SA, Elobeid MA, Virk P, Al-Olayan EM, et al. Olive oil oleuropein has anti-breast cancer properties with higher efficiency on ER-negative cells. *Food and Chemical Toxicology*. 2013; 53: 310–316. <https://doi.org/10.1016/j.fct.2012.12.009> PMID: 23261678
66. Sherif IO, Al-Gayyar MMH. Oleuropein potentiates anti-tumor activity of cisplatin against HepG2 through affecting proNGF/NGF balance. *Life Sciences*. 2018; 198: 87–93. <https://doi.org/10.1016/j.lfs.2018.02.027> PMID: 29476769
67. Papanikolaou C, Melliou E, Magiatis P. Olive Oil Phenols. In *Functional Foods*, (IntechOpen). 2019. pp 9. <http://dx.doi.org/10.5772/intechopen.81394>
68. Gálvez M, Martín-Cordero C, Ayuso MJ. Pharmacological Activities of Phenylpropanoids Glycosides. In *Studies in Natural Products Chemistry*, Elsevier. 2006. pp. 675–718.
69. Khan MS, Abul Qais F, Ahmad I, Hussain A, Alajmi MF. Genotoxicity inhibition by *Syzygium cumini* (L.) seed fraction and rutin: understanding the underlying mechanism of DNA protection. *Toxicology Research*. 2018; 7: 156–171. <https://doi.org/10.1039/c7tx00269f> PMID: 30090571