Mexican sunflower (Tithonia diversifolia, Asteraceae) volatile oil as a selective inhibitor of Staphylococcus aureus nicotinate mononucleotide adenylyltransferase (NadD)

Mexican sunflower (*Tithonia diversifolia*, Asteraceae) volatile oil as a selective inhibitor of *Staphylococcus aureus* nicotinate mononucleotide adenylyltransferase (NadD)

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Abstract

Tithonia diversifolia, well-known as Mexican sunflower, is an invasive shrub growing in tropical areas of South America, Asia and Africa where it is used as a traditional medicine, ornamental plant and green biomass to improve soil fertility. Given the traditional uses in the treatment of skin infections, we have first analysed the chemical composition and the antimicrobial effects of the essential oil hydrodistilled from inflorescences of *T. diversifolia*. For the purpose the inhibition

zones against a panel of pathogens were measured by the agar diffusion method. In addition, we evaluated the inhibitory effects on several NaMN/NMN adenylyltransferases, which are essential enzymes for NAD biosynthesis in most bacterial pathogens, and also tested the inhibition on the mammalian orthologue enzymes as a promising way to identify novel natural antibiotics. To complete the screening of biological effects, the antioxidant capacity and antiproliferative effects on human tumor cells were evaluated using the DPPH, ABTS, FRAP, and MTT methods. Results showed that *T. diversifolia* essential oil was mostly active against *Staphylococcus aureus* with a halo of 14 mm. The essential oil selectively inhibited *in vitro* the pure NAD biosynthetic enzyme NadD from *S. aureus* (IC_{50} of ~60 μg/mL), with basically none or only minor effects on mammalian orthologue enzymes. Finally, the essential oil displayed significant cytotoxic effects on A375, MDA-MB 231, HCT 116 and T98G tumor cells with IC_{50} values of 3.02, 3.79, 3.46 and 12.82 μg/mL, respectively, and noticeable radical scavenging activity on DPPH and ABTS radicals, with IC_{50} values of 108.8 and 41.7 μg/mL, respectively.

Keywords: *Tithonia diversifolia*, essential oil, antimicrobial, *S. aureus* NadD, *Mus musculus* NMNATs, cytotoxicity.

1. Introduction

The genus *Tithonia* Desf. Ex Jussieu (Asteraceae, tribe Heliantheae), native to both Mexico and Central America, includes eleven species (Chagas-Paula et al., 2012). It is mainly represented by shrubs or trees with aerial parts covered by glandular trichomes and bearing solitary yellow inflorescence heads (capitula). Currently, among the several species within the genus that are distributed and cultivated around the world, *T. diversifolia* (Hemsl.) A. Gray is definitely the most studied. The plant, also known as Mexican sunflower, reaches heights of up to 3 m and is nowadays distributed in tropical areas of South America, Asia and Africa (Chagas-Paula et al., 2012). The plant flowers and produces seeds throughout the year. Because of its stoutness and showy capitula,

T. diversifolia is widely used as an ornamental plant. As it grows very quickly, it is frequently invasive of agricultural and non-agricultural lands (Ayeni et al., 1997), and potentially useful as a green biomass to improve soil fertility (Jama et al., 2000). Also attempts to use *T. diversifolia* as a daily supplement in poultry diet have been made in Nigeria (Ekeocha, 2012).

In tropical and sub-tropical regions, thanks to its abundant availability, *T. diversifolia* has become commonly used in the traditional medicine by local ethnic groups (Heinrich, 2000). In the American traditional medicine the juice obtained from stems and leaves is used for the treatment of abscesses (Játem-Lásser et al., 1998), hematomas, and muscular cramps, whereas taken orally is employed against malaria (Heinrich et al., 1998). Antique civilization such as Maya used the powder obtained by crashing toasted leaves to treat various skin diseases (Heinrich, 2000). In Africa, *T. diversifolia* is used orally or to make baths to treat microbiological infections in sexual organs (Kamatenesi-Mugisha et al., 2008). The infusion of leaves, in addition to the treatment of malaria, represents an antidote to snake bites (Owuor and Kisangau, 2006, Njoroge and Bussman, 2006). In Asia, the leaf aqueous extract is taken for the treatment of diabetes (Miura et al., 2005; Takahashi, 1998). The effectiveness of some traditional uses above reported was demonstrated by rigorous scientific studies (Passoni et al., 2013).

Focusing on the secondary metabolites from *T. diversifolia*, most reports highlight the sesquiterpene lactones as the prominent group. These compounds are indeed chemotaxonomic markers of the *Asteraceae* family (Ferreira et al., 2005). Among them, the so called tagitinins are the most representatives in *T. diversifolia*, with tagitinin C being the lead compound. These germacranolides have been reported as responsible for the wide spectrum of pharmacological activity attributed to *T. diversifolia*, mainly encompassing anti-inflammatory/analgesic (Owoyele et al., 2004), and antimalarial (Goffin et al., 2002) properties. Tagitinins are bitter-tasting compounds, both inhibiting proliferation of human malignant glioblastoma cells (Liao et al., 2011) and exerting allelopathic, antifeedant, and insecticidal activity (Ambrósio et al., 2008; Castaño-Quintana et al., 2013). Other classes of secondary metabolites are given by diterpenoids, flavonoids, and chlorogenic acid

derivatives. As far as the volatile fraction is concerned, only a few studies are available, reporting the monoterpenes α-pinene, β-pinene, limonene, and (Z)-β-ocimene as the major essential oil constituents of *T. diversifolia* inflorescences (Lawal et al., 2013; Gbolade et al., 2008; Menut et al., 1992). Besides, to our knowledge, there is no report on the biological activities of *T. diversifolia* essential oil.

Therefore, in the present work, we investigated the *in vitro* biological effects of *T. diversifolia* essential oil, namely its antibacterial and antioxidant activity, and its cytotoxicity on human tumor cells. For the purpose, spontaneous plants from Western highlands of Cameroon, where *T. diversifolia* is naturalised, were utilized. The different biological activities were assessed by agar disc-diffusion, DPPH, ABTS, FRAP, and MTT methods. Furthermore, we evaluated the inhibitory effects of the essential oil on nicotinate mononucleotide adenylyltransferase (NadD, EC 2.7.7.18), a key NAD biosynthetic enzyme shared by most bacterial pathogens (Sorci et al., 2009; Huang et al., 2010; Rodionova et al., 2015), as well as on the three orthologue isozymes of the mammalian host that are known as nicotinamide mononucleotide adenylyltransferase (NMNAT, EC 2.7.7.1) (Sorci et al., 2007; Orsomando et al., 2012; Mori et al., 2014). All enzymes of this class, despite different acronyms and EC classification that are somehow justified by enzyme-distinctive substrate specificities, share common structure, catalytic mechanism, and essentiality for cell/organism survival (Magni et al., 2004; Zhai et al., 2009; Pankiewicz et al., 2015). They thus represent very promising druggable targets for developing novel drugs with either antibiotic or antiproliferative activity (Magni et al., 2009; Petrelli et al., 2011). The findings of this work may provide new insights into the industrial uses of Mexican sunflower as a renewable biomass to be exploited for pharmaceutical applications.

2. Materials and Methods

2.1. Plant material

Flowerheads (capitula) of *T. diversifolia* were harvested in Dschang, West Province of Cameroon c/o Campus of Dschang University (N 05°26'18", E 10°04'07", 1450 m a.s.l.), in March 2013. A voucher specimen was authenticated by plant taxonomist Mr. Nana and deposited at the National Herbarium of Yaounde, Cameroon, under the code 10196/HNC. Flowerheads were dried at ≈25 °C in the shade for seven days before undergoing hydrodistillation.

2.2. Hydrodistillation

Dry flowerheads (capitula) (350 g) were reduced into small pieces, then subjected to hydrodistillation in a Clevenger-type apparatus for 4 h using 6 L of deionized water. The essential oil yield (0.04%) was determined on a dry-weight basis (w/w). Once obtained, the oil was dried (Na_2SO_4), stored into an amber glass flask, and kept at -20°C before chemical analysis and biological experiments.

2.3. *Chemicals*

The analytical standards *n*-hexanal, n-nonane, α-pinene, camphene, benzaldehyde, β-pinene, 1-octen-3-ol, *p*-cymene, limonene, γ-terpinene, terpinolene, linalool, *trans*-thujone, *trans*-pinocarveol, terpinen-4-ol, α-terpineol, myrtenol, verbenone, *n*-decanal, geraniol, geranial, (*E*)-caryophyllene, α-humulene, (*E*)-β-ionone, (*E*)-nerolidol, caryophyllene oxide, *n*-hexadecanoic acid were purchased from SigmaAldrich (I-Milan) and used for identification of some peaks; (*E*)-Phytol was previously isolated from *Onosma echioides* (Maggi et al., 2009). A mixture of *n*-alkanes (C_8-C_{30}) was purchased from Supelco (Bellefonte, PA) and used to calculate the temperature-programmed retention indices of chromatographic peaks. *n*-Hexane was purchased from Carlo Erba (I-Milan).

2.4. Chemical analysis of essential oil

Gas chromatographic separation of *T. diversifolia* of volatiles was achieved on an Agilent 6890N gas chromatograph coupled to a 5973N mass spectrometer. For the purpose a HP-5 MS (5% phenylmethylpolysiloxane, 30 m, 0.25 mm i.d., 0.1 μm film thickness; J & W Scientific, Folsom) capillary column was used with the following temperature programme: 5 min at 60°C, subsequently 4°C/min up to 220°C, then 11°C/min up to 280°C, held for 15 min, for a total run of 65 min.

Injector and detector temperatures were 280°C. The carrier gas was He, with a flow rate of 1 mL/min. The split ratio employed was 1:50. Acquisition of mass spectra (*m/z* 29–400) was in electron-impact (EI) mode with an ionization voltage of 70 eV. Before injection, the essential oil was diluted 1:100 in *n*-hexane, then 2 µL of the solution were injected into GC-MS system. For identification of essential oil components co-injection with the above standards was used, together with correspondence of retention indices and mass spectra with respect to literature data (Adams, 2007; NIST 08, 2008; FFNSC2, 2012). Semi-quantification of essential oil components was made by peak area normalisation without calculating GC response factors.

2.5. Antibacterial activity

T. diversifolia essential oil was assayed by agar disc diffusion method against a panel of bacterial species including *Staphylococcus aureus* ATCC 25923 (American Type Culture Collection, Rockville, MD, USA), *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212 following the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2009). Strains were maintained overnight at 37 °C in blood agar plates (Oxoid, Basingstoke, UK). Tested microorganisms (1-2 x 10^8 cells per mL in saline) were spread on the media plates using a sterile cotton swab. Paper discs of 6 mm in diameter were placed on the surface of inoculated plates and spotted with 10 µL of the essential oil. The plates were incubated 24 h at 35 ± 1 °C. The inhibition zones were measured with a calliper. A reading of more than 6 mm meant growth inhibition. The fluoroquinolone ciprofloxacin (5 µg disc) was used as a reference antibiotic. α-Pinene, the most prevalent compound into the essential oil, was also added to the series to test its activity in the pure form (10 µL per disc). The inhibition on *S. aureus* was also investigated by the microdilution method following the international guidelines (CLSI, 2009). Briefly, two-fold serial dilutions of mixture in Cation Adjusted Mueller Hinton Broth was set in 96-well plates starting from 8 mg/l. An equal volume of the microbial inoculum (10^6 cfu/ml), obtained by direct colony suspension of an overnight culture, was added to each well of the microtiter plate containing 0.1 ml of the serially diluted test oil. After incubation for 18–24 h at 35 °C, in normal

atmosphere, the Minimum Inhibitory Concentration (MIC) was calculated as the lowest concentration of the essential oil inhibiting the growth of the bacterium. All microbial tests were done in triplicate.

2.6. Enzyme inhibition assay

Selected NaMN/NMN adenylyltransferase enzymes were chosen to represent either pathogenic bacteria or their mammalian hosts. Pure recombinant NadD from *Staphylococcus aureus* subsp. *aureus* N315 was a generous gift from Dr. Andrei Osterman (Sanford Burnham Prebys Medical Discovery Institute, La Jolla, Ca, USA). The three *Mus musculus* isozymes NMNAT1, NMNAT2, and NMNAT3 were obtained after bacterial overexpression and purification as previously described (Orsomando et al., 2012). Enzyme rates were measured by using two equivalent spectrophotometric coupled methods, namely a continuous assay based on detection at 340 nm of the NADH formed (Balducci et al., 1995), and a discontinuous assay based on detection at 620 nm of the phosphate formed (Vitali et al., 2015). The reaction mixtures, in addition to the buffer, substrates, and ancillary system reactants indicated in Fig. 1 legend, contained 1.5-4 mU/mL of either *S. aureus* NadD or one of the three mammalian NMNAT isoforms. The essential oil from *T. diversifolia* was previously diluted in DMSO and then added to the reaction mixtures at 20-160 µg/mL final concentration. Blank mixtures without the oil but with equal amounts of DMSO were set in parallel and their rates fixed as 100% activity. Each enzyme was preincubated with the oil for 5 min at 37 °C, and then reactions were started by adding NMN for mammalian NMNATs or NaMN for bacterial NadD. Measured rates were linear under these conditions for at least 20 min.

2.7. Antioxidant activity

The antioxidant activity of *T. diversifolia* essential oil was measured by determining the hydrogen donating or radical scavenging ability, using the stable radical DPPH. The assay was assessed on a microplate analytical assay following a previously-described protocol (Srinivasan et al., 2007). Total radical scavenging capacity of the essential oil was measured by the ABTS assay modified as by Re et al. (1999), for application to a 96-well microplate assay. The ferric reducing antioxidant

power (FRAP assay) was carried out according to the procedure described by Müller et al. (2011), by monitoring the reduction of Fe^{3+}-tripyridyl triazine (TPTZ) to blue-coloured Fe^{2+}-TPTZ. The ability of *T. diversifolia* essential oil to scavenge the different radicals in all assays was compared to Trolox used as positive control and expressed as tocopherol-equivalent antioxidant capacity μmol TE/g of product. Each experiment was repeated at least three times.

2.8. MTT assay

A375 (human malignant melanoma cells) and MDA-MB 231 cells (human breast adenocarcinoma cells) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS). HCT116 cells (human colon carcinoma cells), were cultured in RPMI1640 medium with 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and supplemented with 10% HI-FBS. T98G cells (human glioblastoma multiforme cells) were cultured in Eagle's minimum essential medium (EMEM) with 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 μg/mL streptomycin, and supplemented with 10% HI-FBS. Cells were cultured in a humidified atmosphere at 37 °C in presence of 5% CO_2. The MTT assay was used as a relative measure of cell viability. Cell-viability assays were carried out as described (Quassinti et al., 2013). Briefly, cells were seeded at the density of 2×10^4 cells/mL. After 24 h, samples were exposed to different concentrations of essential oil (0.78-200 μg/mL). Cells were incubated for 72 h in a humidified atmosphere of 5% CO_2 at 37 °C. Cisplatin was used as the positive control (0.05-20 μg/mL). At the end of incubation, each well received 10 μL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) (5 mg/ml in phosphate-buffered saline, PBS) and the plates were incubated for 4 h at 37 °C. The extent of MTT reduction was measured spectrophotometrically at 540 nm using a Titertek Multiscan microElisa (Labsystems, FI-Helsinki). Experiments were conducted in triplicate. Cytotoxicity is expressed as the concentration of compound inhibiting cell growth by 50% (IC_{50}).

The IC_{50} values were determined with GraphPad Prism 4 computer program (GraphPad Software, S. Diego, CA, USA).

3. Results and Discussion

3.1. Essential oil analysis

The composition of the essential oil distilled from flowerheads of *T. diversifolia* is reported in Table 1. The chemical profile, obtained by GC-MS, was very complex, with about 250 peaks detected. Of these, 161 were identified, accounting for 87.3% of the total area. Eighteen volatile components were present in relative percentages equal or above 1%, while the remaining (143 components) was present in low relative percentages (below 1%). The oil was mainly composed of monoterpenes (46.9%) with hydrocarbons and oxygenated compounds in similar amounts (24.7 and 22.2%, respectively). Among them, α-pinene (13.7%), limonene (7.6%), and *cis*-chrysanthenol (6.2%) were the most representatives. Sesquiterpenes (23.4%) were the second group characterizing the essential oil, with oxygenated compounds more abundant than hydrocarbons (14.1 and 9.3%, respectively). Main representatives of these classes were spathulenol (3.5%) and α-copaene (3.7%). Aliphatics were a minor fraction of the oil (13.4%), with aldehydes (5.3%), saturated and unsaturated hydrocarbons (3.5%), and fatty acids (3.1%) as the most abundant groups. They were mainly represented by *n*-pentadecanal (1.6%), 1-pentadecene (1.7%) and *n*-hexadecanoic acid (3.0%), respectively.

The composition herein reported showed some relevant differences with respect to previous works. In particular, the essential oil examined was poor of (*Z*)-β-ocimene, which instead was reported as one of the major volatile components of flowers (Menut et al., 1992). On the other hand, the sample examined by us showed significantly lower levels of α-pinene with respect to samples from South Africa and Cameroon previously analysed, where the relative percentages ranged from 61.0 to 72.8% (Lawal et al., 2012; Menut et al., 1992). Again, relevant differences in composition were found with respect to sample from Nigeria (Moronkola et al., 2007), where the sequiterpenes

germacrene D (20.3%), β-caryophyllene (20.1%) and bicyclogermacrene (8.0%) were the most abundant constituents. Another substantial difference with literature data was the higher number of volatiles identified in this study (161 compounds) while a maximum of 57 constituents were previously reported (Moronkola et al., 2007). This makes difficult to correlate the chemical composition with the biological activities displayed by the oil. By the way, the differences in composition might be explained by the different geographic origin, altitude, genetics and processing (e.g. dry vs fresh samples undergoing distillation) of the examined sample.

3.2. Antimicrobial activity

Essential oil was mostly active against *Staphylococcus aureus* (inhibition zone of 14 mm), but showed a moderate to low activity also against *E. faecalis* and *E. coli* (inhibition zones of 8 and 9 mm, respectively) (Table 2). Results from the microdilution test put in evidence a MIC value of 2 mg/ml on *S. aureus*. As stated above, the high heterogeneity of the oil composition made it difficult to point to few major components as responsible of the observed activity. As a matter of facts, we tested the major component α-pinene. Almost 10 mg of pure compound were not able to exert any inhibitory effect. The relative amount of α-pinene into the essential oil (13.7%) might correspond to an absolute amount of 1.37 mg, given that about 10 mg of essential oil was spotted onto the paper disc in the diffusion test. Hence α-pinene was not a significantly active part of the essential oil. According to previous reports (Aggarwal et al., 2002; van Vuuren and Viljoen, 2007), also the relative amount of limonene (7.6%) could not have added significantly to the observed activity. Other minor components, such as *n*-hexadecanoic acid, may have contributed to some extent. However, no other pure compound but α-pinene was tested in the present work.

3.3. Enzyme inhibition tests

The choice of *S. aureus* NadD (*Sa*NadD) as antibacterial target was suggested by the measured inhibition of growth observed in disc diffusion test (Table 2).

Furthermore, we tested the functionally related enzymes of the mammalian host, *i.e.* the three NMNAT isoforms to assess the selectivity of the oil. For this purpose, we chose murine NMNAT

isoforms, available and largely studied in our laboratory, as representative of the mammalian hosts, being highly related and conserved, both structurally and functionally, to the corresponding human isozymes (Orsomando et al., 2012).

Overall these NaMN/NMN adenylyltransferases catalyze the same reaction but are distinguished for their mononucleotide substrate preference. Indeed, NadD strictly catalyzes the conversion of nicotinate mononucleotide (NaMN) and ATP into deamido-NAD and pyrophosphate, while all NMNAT isozymes can also use the amidated substrate NMN to form NAD directly. The three NMNATs in mammals arise from three distinct genes and are endowed with distinct oligomeric structure, subcellular localization, and tissue distribution (Magni et al., 2004; Zhai et al., 2009; Sorci et al., 2007). Their apparent redundancy is likely functional to keep appropriately compartmented NAD levels within different organelles, but also plays important roles at the mammalian organism level. Anyway, both NadD and NMNATs are individually vital in the corresponding organisms because essential for cellular NAD synthesis, as demonstrated by gene deletion, targeted protein degradation, and knocking down experiments (Sorci et al., 2013; Rodionova et al., 2014; Gerdes et al., 2002; Conforti et al., 2011; Gilley et al., 2015). This provides a valuable standpoint for therapies based on selective drug targeting of the enzymes within this class, for which no natural products inhibitors have been reported yet, and prompted us to investigate on the biological effects of the essential oil from *T. diversifolia*.

The results of our *in vitro* assays are shown in Fig. 1. Enzyme mixtures containing 80 μg/mL of essential oil showed ~70% inhibition of ~~*S. aureus* *Sa*~~NadD and ~50% inhibition of murine NMNAT2 compared to their blank controls, with no substantial effect on the other two mammalian isoforms (left panel).

This lack of inhibition in parallel assays of NMNAT1 and NMNAT3 demonstrated that the enzyme inhibition observed for NadD (and NMNAT2) is not arising from aspecific protein binding and structural unfolding, as it could occur for an highly heterogeneous mixture of components, but is triggered by selective inhibition.

Furthermore, on both inhibited enzyme targets, a dose dependent inhibitory effect was evidenced (right panel), yielding to a similar IC_{50} value of 60-70 μg/mL. Of note, all data in Fig. 1 refer to assays carried out at substrates concentration just above the corresponding K_m, but very similar results were also achieved using saturating concentration of both substrates (not shown), thus suggesting a likely non competitive inhibition mechanism exerted by some oil component(s) yet to be identified. Among the list presented in Table 1, most abundant are both α-pinene and limonene, that were further tested individually against all enzymes above under similar assay conditions. As a result, no inhibition was observed with both compounds (not shown), despite being tested at 20 □g/mL, *i.e.* a far higher concentration than predicted in the oil mixture.

Two main conclusions could be drawn from these data. First, unlike our previous report on *T. ammi* (Vitali et al., 2015), the *T. diversifolia* essential oil contains potential inhibitors of vital NAD biosynthetic enzymes, and peculiarly shows selectivity versus NadD, which is unique and indispensable for cell survival in most bacterial pathogens. These molecules, once identified, will then represent valuable novel antibacterials from natural sources.

Second, such potential bioactive components ~~drugs~~ contained in the *T. diversifolia* oil mixture should be highly potent, having ruled out that the antibacterial effect is attributable the most abundant α-pinene and limonene components of the mixture. We estimate, based on the relative abundance of single compounds (see Table 1), that the observed inhibition is due to essential oil components present in the mixture at sub-nanomolar concentrations. However, it is important to underline that the low correlation between the MIC value obtained on *S. aureus* and *Sa*NadD inhibition is very likely due to a lack of cell permeability with this essential oil (Fernicola et al., 2015) or, otherwise, to specific bacterial defence mechanisms. Thus, further studies are in progress in our group to improve the bacterial inhibition of the oil by combination with a cell membrane penetration enhancer.

From a pharmacological point of view, the observed parallel targeting of NMNAT2 in the mammalian host organism appears scarcely significant, being this isozyme ~~almost exclusively~~

311 located in neuronal tissues almost exclusively, and ~~surely~~ less relevant with respect to the most
312 abundant, ubiquitous, and catalytically efficient NMNAT1. ~~Moreover,~~ On the other hand, the
313 identification of selective inhibitors ~~of~~ toward NMNAT2 could aid ~~the~~ crystallization of this
314 enzyme~~, being~~ that represents the only ~~human~~ mammalian isoform still missing a 3D structure
315 definition.

316 *3.4. Antioxidant activity*

317 In the present study three antioxidant assays, DPPH free radical scavenging activity, ABTS radical
318 cation scavenging activity and ferric reducing antioxidant power (FRAP) were applied to accurately
319 evaluate the antioxidant properties of *T. diversifolia* essential oil. The results, expressed as IC_{50} and
320 in absolute terms (i.e., μmol trolox equivalent (TE)/g), are reported in Table 3. *T. diversifolia*
321 essential oil showed significant antioxidant activity, with IC_{50} values of only 30 times lower than
322 that of trolox used as control for DPPH (108.8 μg/ml**)** and ABTS+ (41.7 μg/ml). Different studies
323 have indicated that the antioxidant activity is associated with electron donation capacity, reflecting
324 the reducing power, of bioactive constituents (Siddhuraju et al., 2002; Arabshahi-Delouee and
325 Urooj, 2007) and the results obtained in FRAP assay (Table 3) show also in this case significative
326 antioxidant reducing potential for the *T. diversifolia* essential oil. In general in the plant essential
327 oils, monoterpene hydrocarbons and oxygenated monoterpenes are mainly responsible for the
328 antioxidant potential (Ruberto and Baratta, 2000; Miguel, 2010). According to our analysis,
329 monoterpene hydrocarbons (24.7%) and oxygenated monoterpenes (22.2%) were the main
330 components of the mexican sunflower essential oil and they probably contribute significantly to the
331 antioxidant activity observed. Based on our previous study (Fogang et al., 2012), the presence in
332 high percentage of some constituents such as α-pinene (13.7%) and limonene (7.6%), (Table 1)
333 may contribute to the total antioxidant activity observed. Also the free radical scavenging activity
334 of the essential oil may have resulted from the synergistic action of the complex mixture of minor
335 components detected. Our data suggest the possibility of using the essential oil of mexican
336 sunflower as a natural preservative.

337 *3.5. Cytotoxic activity*

338 The antiproliferative potential of *T. diversifolia* essential oil was further determined using the MTT
339 assay against A375 human malignant melanoma cell line, MDA-MB 231 human breast
340 adenocarcinoma cell line, HCT116 human colon carcinoma and T98G human glioblastoma
341 multiforme cell line. As indicated in Table 4, the essential oil demonstrated a promising result, with
342 cell inhibition observed at 72 h of incubation. The results suggested that *T. diversifolia* essential oil
343 inhibits the proliferation of A375, MDA-MB 231, HCT116 and T98G cells in a dose-dependent
344 manner. Seventy-two-hour incubation of cells with *T. diversifolia* essential oil resulted in an IC_{50}
345 values of 3.02, 3.79, and 3.46 µg/mL for A375, MDA-MB 231, and HCT116 cells, respectively.
346 These IC_{50} values are close to those reported for cisplatin, an anticancer chemotherapy drug which
347 was used as the positive control (IC_{50} values of 2.29 and 2.34 µg/mL on MDA-MB 231 and
348 HCT116, respectively). Essential oil resulted less active on T98G cells with IC_{50} value of 12.82
349 µg/mL. However, this value provided by an essential oil is worthy of consideration since
350 glioblastoma is one of the most lethal and particularly aggressive and invasive human brain tumors
351 in humans so that it is more resistant to most of current chemotherapy regimens (Karmakar et al.,
352 2006).

353 The observed cytotoxicity of *T. diversifolia* essential oil was not specific toward a cancer cell line.
354 In this first report of the cytotoxic activity of *T. diversifolia* essential oil, a compound or few
355 compounds do not emerge from the composition of essential oil that can be responsible for the
356 cytotoxic activity on human tumor cell lines. Our previous study has demonstrated that α-pinene
357 and limonene showed antiproliferative activity on same cell lines above tested with IC_{50} values
358 ranging from 27.3 to 63.1 µg/mL and 18.4 to 124.0 µg/mL, respectively (Fogang et al., 2012). In
359 the same range of concentrations, α-pinene exerts antiproliferative activity on MCF-7 (mammary
360 adenocarcinoma), MDA-MB-231 (mammary adenocarcinoma), MDA-MB-468 (mammary
361 adenocarcinoma), and UACC-257 (malignant melanoma) (Bansal et al., 2007). Limonene also
362 induces apoptosis in LS174T colon cancer cells and in lymphoma cell line (35 µg/mL, IC_{50}) (Jia et

al., 2013; Manuele et al., 2008), moreover it shows antitumor activity on lung adenocarcinoma A549 (0.098 µL/mL, IC_{50}) and hepatocarcinoma HepG2 (0.150 µL/mL, IC_{50}) (Manassero et al., 2013). Spathulenol was reported weakly active on human epidermoid carcinoma (KB) and inactive on human breast cancer (BC) and human small cell lung cancer (NCIH187) cell lines (Prawat et al. 2013). To our knowledge, data reporting the cytotoxic activity of *cis*-chrysanthenol, epoxycyclooctane, and α-copaene are missing. However, the concentrations of α-pinene (13.1%), limonene (7.6%), and cis chrysanthenol (6.2%) cannot fully explain the cytotoxic activity of *T. diversifolia* essential oil, which means that the other minor compounds contributed to the activity of the essential oil or a synergism between the compounds increases their specific antiproliferative activity.

In the plant screening program of the National Cancer Institute (NCI) of the USA, a crude oil is generally considered promising as cytotoxic agent if the IC_{50} value, following incubation between 48 and 72 h, is less than 20 µg/mL (Boik 2001). *T. diversifolia* essential oil showed an excellent inhibitory activity against the three human tumor cell lines with IC_{50} values comparable to those of the positive control, which in turn deserves further investigation. Worthy of mention was the antiproliferative activity showed on glioblastoma that is particularly resistant to many chemotherapics. The major drawbacks of chemotherapy for treatment of glioblastoma are the incapability of many drugs to cross the blood-brain barrier. In this regard, the mexican sunflower essential oil, being a mixture of many small lipophilic molecules, may represent a potential treatment of these invasive tumors.

4. Conclusions

T. diversiflora is a tropical shrub cultivated in many developing countries where it grows very quickly so that it becomes invasive of agricultural and non-agricultural lands. Therefore, it may be considered as a green biomass to be used as a renewable source of essential oil to be exploited on an industrial level. Results of this work showed that *S. aureus* cell growth, as well as its essential NAD

biosynthetic enzyme NadD, are both inhibited by *T. diversifolia* essential oil, and that these effects are not attributable to at least two ~~one of its~~ major components, α-pinene and limonene. Further studies are required to verify that the *S. aureus* growth suppression is due to an on-target activity of the *T. diversifolia* essential oil mixture. Enzyme inhibition also appears to selectively target NadD from this bacterial pathogen, at least *in vitro*, since none or minor effects were observed in parallel assays with mammalian orthologue enzymes. ~~On this respect, it must be emphasized that the three known mammalian NMNATs arise from distinct genes and show distinct oligomeric structure, subcellular localization, and tissue distribution; their redundancy is thought to be functional to keep appropriate NAD compartmentation at both cell and organism levels. Nonetheless, from a pharmacological point of view, the observed inhibition of NMNAT2 appears scarcely significant in the economy of the whole mammalian organism, being this isozyme almost exclusively located in neuronal tissues and surely less relevant with respect to the most abundant, ubiquitous, and catalytically efficient NMNAT1. On the other hand, identification of specific inhibitors of NMNAT2 could at least aid future crystallization studies, being this isozyme form among the three the only still missing a structural definition.~~ The mexican sunflower essential oil revealed to be also a potent cytotoxic agent on tumor cells, with activity comparable in some cases to that of the anticancer drug cisplatin. ~~Worthy of mention was the antiproliferative activity showed on glioblastoma that is particularly resistant to many chemotherapics. The major drawbacks of chemotherapy for treatment of glioblastoma are the incapability of many drugs to cross the blood-brain barrier. In this regard, the mexican sunflower essential oil, being a mixture of many small lipophilic molecules, may represent a potential treatment of these invasive tumors. Finally, antioxidant assays suggest the possibility of using the essential oil of mexican sunflower as a natural preservative due to its noteworthy radical scavenging activity.~~

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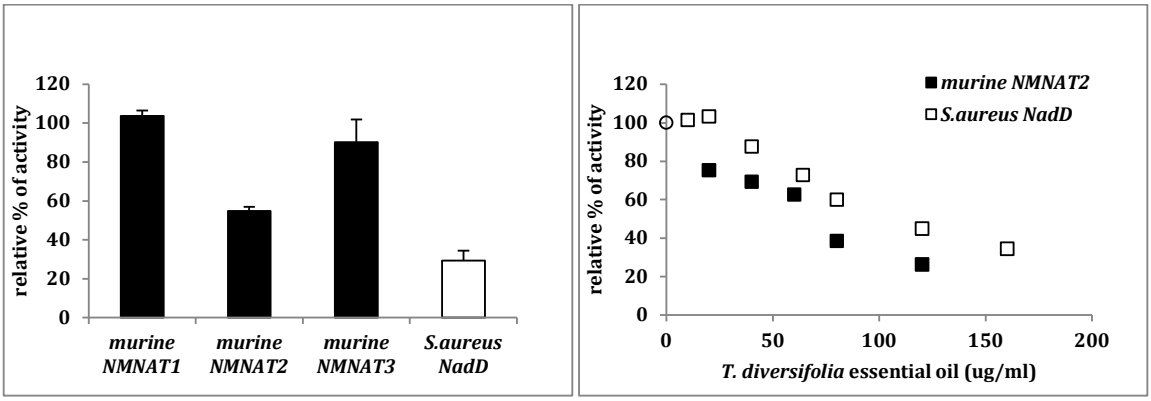

Figure 1. ***In vitro*** **effect of** ***Tithonia diversifolia*** **essential oil on indispensable NaMN/NMN adenylyltransferases from various sources**. Data reported were from either the continuous (black bars and squares) or the discontinuous (white bar and squares) assay described in M&M, carried out at 80 µg/ml essential oil concentration (left panel) or variable oil concentration (right panel). The continuous assay of the three individual murine NMNAT isoforms was carried out in 30 mM HEPES buffer, pH 7.5, 0.04 mM NMN, 0.08 mM ATP, 12.5 mM $MgCl_2$, 1% (v/v) DMSO, 75 mM ethanol, 30 mM semicarbazide, 12.5 U/ml yeast alcohol dehydrogenase, and 0.5 mg/ml BSA. The discontinuous assay of *S. aureus* NadD was carried out in 100 mM HEPES buffer, pH 7.5, 0.05 mM NaMN, 0.1 mM ATP, 10 mM $MgCl_2$, 1% (v/v) DMSO), 2 U/ml yeast inorganic pyrophosphatase, and 0.1 mg/ml BSA. Each enzymatic assay was at least in duplicate, and the activity values were reported as percentages relative to parallel blank controls.

Table 1. Chemical composition of the essential oil from flowerheads of *Tithonia diversifolia*.

N.	Component[a]	RI calc.[b]	RI lit[c]	%[d]	ID[e]
1	*n*-hexanal	801	801	0,2	Std
2	1,3-cyclopentadiene, 1,2,5,5-tetramethyl-	837	835	Tr[f]	RI,MS
3	(2*E*)-hexenal	850	846	Tr	RI,MS
4	*n*-hexanol	866	863	Tr	RI,MS
5	2-heptanone	892	892	0,1	RI,MS
6	4-heptenal	893	895	1,0	RI,MS
7	*n*-nonane	899	900	0,1	Std
8	*n*-heptanal	903	902	0,1	RI,MS
9	tricyclene	920	921	Tr	RI,MS
10	α-thujene	925	924	0,1	RI,MS
11	α-pinene	932	932	13,7	Std
12	camphene	945	946	0,1	Std
13	thuja-2,4(10)-diene	951	953	0,3	RI,MS
14	(2*E*)-heptenal	957	947	Tr	RI,MS
15	benzaldehyde	961	952	0,1	Std
16	sabinene	971	969	0,6	RI,MS
17	β-pinene	973	974	0,3	Std
18	1-octen-3-ol	980	974	0,1	Std
19	2,3-octanedione	986	985	Tr	RI,MS
20	6-methyl-5-hepten-2-one	989	981	0,2	RI,MS
21	2-pentyl furan	991	984	2,3	RI,MS
22	*m*-cymene	1004	1002	0,2	RI,MS
23	α-terpinene	1017	1014	0,1	RI,MS
24	1,2,4-trimethyl benzene	1023	1021	Tr	RI,MS
25	*p*-cymene	1026	1024	0,4	Std
26	limonene	1030	1024	7,6	Std
27	(*Z*)-β-ocimene	1043	1032	0,3	RI,MS
28	benzene acetaldehyde	1048	1036	0,1	RI,MS
29	(*E*)-β-ocimene	1052	1044	0,2	RI,MS

30	γ-terpinene	1061	1054	0,3	Std
31	(2*E*)-octen-1-al	1063	1054	0,1	RI,MS
32	*cis*-sabinene hydrate	1069	1065	0,3	RI,MS
33	*cis*-linalool oxide	1075	1067	0,1	RI,MS
34	terpinolene	1087	1086	0,2	Std
35	*p*-cymenene	1089	1089	0,2	RI,MS
36	6-camphenone	1092	1095	0,4	RI,MS
37	*trans*-sabinene hydrate	1096	1098	0,1	RI,MS
38	(*Z*)-6-nonenal	1098	1100	0,6	RI,MS
39	linalool	1100	1095	2,0	Std
40	*n*-nonanal	1105	1100	0,5	RI,MS
41	1,3,8-*p*-menthatriene	1108	1109	Tr	RI,MS
42	*trans*-thujone	1115	1112	Tr	Std
43	6-camphenol	1117	1111	0,1	RI,MS
44	*trans*-*p*-mentha-2,8-dien-1-ol	1120	1119	0,5	RI,MS
45	α-campholenal	1125	1122	1,9	RI,MS
46	*cis*-limonene oxide	1130	1132	0,1	RI,MS
47	*trans*-pinocarveol	1136	1135	0,7	Std
48	*cis*-verbenol	1141	1137	0,6	RI,MS
49	*trans*-verbenol	1144	1140	1,3	RI,MS
50	1,4-dimethyl-3-cyclohexenyl methyl ketone	1151	1145	0,1	RI,MS
51	sabina ketone	1152	1154	0,1	RI,MS
52	lilac aldehyde A	1154	1155	0,1	RI,MS
53	*trans*-pinocamphone	1158	1158	0,6	RI,MS
54	pinocarvone	1161	1160	0,4	RI,MS
55	*cis*-chrysanthenol	1165	1160	6,2	RI,MS
56	*p*-mentha-1,5-dien-8-ol	1168	1166	0,6	RI,MS
57	*cis*-pinocamphone	1171	1172	0,3	RI,MS
58	terpinen-4-ol	1175	1174	1,1	Std
59	*p*-cymen-8-ol	1185	1179	0,5	RI,MS

60	α-terpineol	1188	1186	0,3	Std
61	myrtenal	1191	1195	0,2	Std
62	myrtenol	1193	1194	0,6	RI,MS
63	safranal	1196	1197	0,1	RI,MS
64	verbenone	1205	1204	Tr	Std
65	*n*-decanal	1206	1201	0,5	Std
66	*trans*-carveol	1217	1215	0,9	RI,MS
67	nerol	1231	1227	0,2	RI,MS
68	cumin aldehyde	1238	1238	0,2	RI,MS
69	carvone	1243	1239	0,4	Std
70	β-cyclohomocitral	1255	1254	Tr	RI,MS
71	(4*E*)-decen-1-ol	1258	1259	0,3	RI,MS
72	geraniol	1259	1249	0,3	Std
73	*n*-decanol	1264	1266	0,1	RI,MS
74	phellandral	1271	1271	0,3	RI,MS
75	geranial	1273	1264	0,3	Std
76	α-terpinen-7-al	1281	1283	0,1	RI,MS
77	(2*E*,4*Z*)-decadienal	1294	1292	0,2	RI,MS
78	2-undecanone	1295	1293	0,1	RI,MS
79	*n*-tridecane	1300	1300	0,1	Std
80	2,3,4-trimethyl benzaldehyde	1311	1313	0,1	RI,MS
81	(2*E*,4*E*)-decadienal	1316	1315	0,3	RI,MS
82	*p*-mentha-1,4-dien-7-ol	1330	1325	0,1	RI,MS
83	presilphiperfol-7-ene	1334	1334	0,1	RI,MS
84	α-longipinene	1342	1350	0,1	RI,MS
85	2,3,6-trimethyl benzaldehyde	1350	1352	0,1	RI,MS
86	α-copaene	1370	1374	3,7	RI,MS
87	α-isocomene	1377	1387	0,2	RI,MS
88	7-decen-1-ol acetate	1379	1389	0,1	RI,MS
89	(*E*)-β-damascenone	1382	1383	0,1	RI,MS
90	β-isocomene	1393	1407	0,2	RI,MS

91	α-*cis*-bergamotene	1400	1411	0,1	RI,MS
92	*n*-tetradecane	1400	1400	0,1	Std
93	β-cedrene	1408	1419	0,2	RI,MS
94	(*E*)-caryophyllene	1408	1417	0,2	Std
95	α-*trans*-bergamotene	1432	1432	0,1	RI,MS
96	aromadendrene	1443	1439	0,1	RI,MS
97	α-humulene	1445	1454	0,1	Std
98	*allo*-aromadendrene	1452	1458	0,1	RI,MS
99	geranyl acetone	1454	1453	0,4	RI,MS
100	(*E*)-β-farnesene	1458	1454	0,1	RI,MS
101	selina-4,11-diene	1469	1474*	0,2	RI,MS
102	germacrene D	1473	1484	0,3	RI,MS
103	β-selinene	1477	1489	0,4	RI,MS
104	eremophilene	1480	1482	0,4	RI,MS
105	(*E*)-β-ionone	1482	1487	0,2	Std
106	δ-selinene	1484	1492	0,3	RI,MS
107	α-selinene	1486	1498	0,2	RI,MS
108	*epi*-cubebol	1488	1493	0,1	RI,MS
109	1-pentadecene	1493	1493	1,7	RI,MS
110	(*Z*,*E*)-α-farnesene	1495	1494	0,9	RI,MS
111	β-bisabolene	1505	1505	0,1	RI,MS
112	cubebol	1507	1514	0,1	RI,MS
113	*trans*-calamenene	1517	1521	0,3	RI,MS
114	δ-cadinene	1517	1523	0,3	RI,MS
115	α-calacorene	1535	1544	0,4	RI,MS
116	β-calacorene	1556	1564	0,2	RI,MS
117	(*E*)-nerolidol	1564	1561	1,0	Std
118	spathulenol	1569	1577	3,5	RI,MS
119	caryophyllene oxide	1572	1582	1,2	Std
120	β-copaen-4-α-ol	1578	1590	0,7	RI,MS
121	β-oplopenone	1594	1607	0,7	RI,MS

122	junenol	1605	1618	1,4	RI,MS
123	tetradecanal	1612	1611	0,2	RI,MS
124	humulane-1,6-dien-3-ol	1618	1619	0,3	RI,MS
125	muurola-4,10(14)-dien-1-β-ol	1621	1630	0,4	RI,MS
126	silphiperfol-6-en-5-one	1626	1624	0,2	RI,MS
127	*iso*-spathulenol	1630	1631*	0,2	RI,MS
128	*epi*-α-cadinol	1633	1638	0,5	RI,MS
129	cubenol	1636	1645	0,2	RI,MS
130	β-eudesmol	1640	1649	0,2	RI,MS
131	selin-11-en-4-α-ol	1645	1660	1,1	RI,MS
132	α-cadinol	1647	1652	0,3	RI,MS
133	*cis*-calamenen-10-ol	1653	1660	0,2	RI,MS
134	*trans*-calamenen-10-ol	1661	1668	0,3	RI,MS
135	cadalene	1673	1675	0,1	RI,MS
136	amorpha-4,9-dien-2-ol	1698	1700	0,3	RI,MS
137	2-pentadecanone	1699	1697	0,1	RI,MS
138	n-pentadecanal	1714	1714	1,6	RI,MS
139	γ-costol	1739	1745	0,5	RI,MS
140	eupatoriochromene	1753	1761	0,4	RI,MS
141	α-costol	1763	1773	0,2	RI,MS
142	tetradecanoic acid	1768	1767	0,1	RI,MS
143	*epi*-cyclocolorenone	1776	1774	0,1	RI,MS
144	1-tridecene-3,5,7,9,11-pentayne	1805	1809*	0,1	RI,MS
145	*n*-hexadecanal	1816	1816	Tr	RI,MS
146	2-pentadecanone, 6,10,14-trimethyl-	1846	1845	0,4	RI,MS
147	2-phenyltridecane	1903	1903	Tr	RI,MS
148	(5*E*,9*E*)-farnesyl acetone	1916	1913	0,1	RI,MS
149	methyl hexadecanoate	1928	1921	0,2	RI,MS
150	isophytol	1948	1946	0,1	RI,MS
151	*n*-hexadecanoic acid	1965	1959	3,0	Std
152	methyl linoleate	2097	2095	0,1	RI,MS

153	*n*-heneicosane	2100	2100	0,1	Std
154	(*E*)-phytol	2116	2116	Tr	Std
155	*n*-tricosane	2300	2300	0,6	Std
156	*n*-tetracosane	2400	2400	0,1	Std
157	*n*-pentacosane	2500	2500	0,6	Std
158	methyl docosanoate	2534	2531	Tr	RI,MS
159	*n*-heptacosane	2700	2700	0,1	Std
160	squalene	2837	2847	0,1	RI,MS
161	*n*-nonacosane	2903	2900	Tr	Std

Total identified (%)	87,3
Grouped compounds (%)	
Terpenoids	
Monoterpene hydrocarbons	24,7
Oxygenated monoterpenes	22,2
Sesquiterpene hydrocarbons	9,3
Oxygenated sesquiterpenes	14,1
Diterpenes	0,1
Norisoprenoids	0,4
Aromatics	0,4
Aliphatics	
Fatty acids	3,1
Alkanes, alkenes and alkynes	3,5
Esters	0,5
Alcohols	0,5
Aldehydes	5,3
Ketones	0,5
Others	2,8

[a] Compounds are listed in order of their elution from a HP-5MS column. [b] Linear retention index on HP-5MS column, experimentally determined using homologous series of C_8-C_{30} alkanes. [c] Linear retention index taken from Adams (2007) and/or NIST 08 (2008). [d] Relative percentage values are means of three determinations with a RSD% in all cases below 10%. [e] Identification methods: std, based on comparison with authentic compounds; NMR, based on spectroscopic data; MS, based on comparison with WILEY, ADAMS, FFNSC2 and NIST 08 MS databases; RI, based on comparison of calculated RI with those reported in ADAMS, FFNSC 2 and NIST 08. [f] Tr, % below 0.1%.

Table 2. Antimicrobial activity of *Tithonia diversifolia* essential oil by the diffusion disk method. Each value represent the diameter of the inhibition zone (millimeter) and is the average of three determinations. When appropriate, standard deviation is also indicated (± SD).

	S. aureus ATCC 29213	*E. faecalis* ATCC 29212	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853
Essential oil	14.0 ± 1.0	8.0 ± 0.5	9.0 ± 0.5	6.0 [a]
α-pinene	6.0 [a]	6.0 [a]	6.0 [a]	6.0 [a]
Ciprofloxacin	21.7 ± 1.5	20.3 ± 0.6	30.3 ± 0.6	29.3 ± 1.2

[a] no activity (no inhibition zone diameter)

Table 3. *In-vitro* radical-scavenging activities of the essential oil from *Tithonia diversifolia*.

	DPPH		**ABTS**		**FRAP**
	TEAC μmolTE/g	IC_{50} μg/ml	TEAC μmolTE/g	IC_{50} μg/ml	TEAC μmol TE/g
Essential oil	139.0 ±10.5	108.8 ±4.30	142.0 ±8.20	41.7 ±1.8	167.3 ±11.5
Positive control					
Trolox		3.78 ±0.13		1.48 ±0.2	

Table 4. *In vitro* cytotoxic activity of *Tithonia diversifolia* essential oil.

	Cell line (IC_{50} µg/mL)[a]			
	A375[b]	MDA-MB 231[c]	HCT116[d]	T98G[e]
Essential oil	3.02	3.79	3.46	12.82
95% C.I.[f]	2.64-3.47	3.28-4.38	3.25-3.68	11.56-14.22
Positive control				
Cisplatin	0.40	2.29	2.34	2.07
95% C.I.	0.33-0.46	2.04-2.78	2.14-2.59	1.86-2.23

[a] IC_{50} = The concentration of essential oil/compound that affords a 50% reduction in cell growth (after 72 h of incubation). [b] Human malignant melanoma cell line. [c] Human breast adenocarcinoma cell line. [d] Human colon carcinoma cell line. [e]Human glioblastoma multiforme cell line. [f] Confidence interval.