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note finali coverage

(Article begins on next page)

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

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20
21 **Abstract**

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

27 zones against a panel of pathogens were measured by the agar diffusion method. In addition, we
28 evaluated the inhibitory effects on several NaMN/NMN adenylyltransferases, which are essential
29 enzymes for NAD biosynthesis in most bacterial pathogens, and also tested the inhibition on the
30 mammalian orthologue enzymes as a promising way to identify novel natural antibiotics. To
31 complete the screening of biological effects, the antioxidant capacity and antiproliferative effects on
32 human tumor cells were evaluated using the DPPH, ABTS, FRAP, and MTT methods. Results
33 showed that *T. diversifolia* essential oil was mostly active against *Staphylococcus aureus* with a
34 halo of 14 mm. The essential oil selectively inhibited *in vitro* the pure NAD biosynthetic enzyme
35 NadD from *S. aureus* (IC₅₀ of ~60 µg/mL), with basically none or only minor effects on mammalian
36 orthologue enzymes. Finally, the essential oil displayed significant cytotoxic effects on A375,
37 MDA-MB 231, HCT 116 and T98G tumor cells with IC₅₀ values of 3.02, 3.79, 3.46 and 12.82
38 µg/mL, respectively, and noticeable radical scavenging activity on DPPH and ABTS radicals, with
39 IC₅₀ values of 108.8 and 41.7 µg/mL, respectively.

40

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

43

44 **1. Introduction**

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

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

57 In tropical and sub-tropical regions, thanks to its abundant availability, *T. diversifolia* has become
58 commonly used in the traditional medicine by local ethnic groups (Heinrich, 2000). In the
59 American traditional medicine the juice obtained from stems and leaves is used for the treatment of
60 abscesses (Játem-Lászer et al., 1998), hematomas, and muscular cramps, whereas taken orally is
61 employed against malaria (Heinrich et al., 1998). Antique civilization such as Maya used the
62 powder obtained by crashing toasted leaves to treat various skin diseases (Heinrich, 2000). In
63 Africa, *T. diversifolia* is used orally or to make baths to treat microbiological infections in sexual
64 organs (Kamatenesi-Mugisha et al., 2008). The infusion of leaves, in addition to the treatment of
65 malaria, represents an antidote to snake bites (Owuor and Kisangau, 2006, Njoroge and Bussman,
66 2006). In Asia, the leaf aqueous extract is taken for the treatment of diabetes (Miura et al., 2005;
67 Takahashi, 1998). The effectiveness of some traditional uses above reported was demonstrated by
68 rigorous scientific studies (Passoni et al., 2013).

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

79 derivatives. As far as the volatile fraction is concerned, only a few studies are available, reporting
80 the monoterpenes α -pinene, β -pinene, limonene, and (Z)- β -ocimene as the major essential oil
81 constituents of *T. diversifolia* inflorescences (Lawal et al., 2013; Gbolade et al., 2008; Menut et al.,
82 1992). Besides, to our knowledge, there is no report on the biological activities of *T. diversifolia*
83 essential oil.

84 Therefore, in the present work, we investigated the *in vitro* biological effects of *T. diversifolia*
85 essential oil, namely its antibacterial and antioxidant activity, and its cytotoxicity on human tumor
86 cells. For the purpose, spontaneous plants from Western highlands of Cameroon, where *T.*
87 *diversifolia* is naturalised, were utilized. The different biological activities were assessed by agar
88 disc-diffusion, DPPH, ABTS, FRAP, and MTT methods. Furthermore, we evaluated the inhibitory
89 effects of the essential oil on nicotinate mononucleotide adenylyltransferase (NadD, EC 2.7.7.18), a
90 key NAD biosynthetic enzyme shared by most bacterial pathogens (Sorci et al., 2009; Huang et al.,
91 2010; Rodionova et al., 2015), as well as on the three orthologue isozymes of the mammalian host
92 that are known as nicotinamide mononucleotide adenylyltransferase (NMNAT, EC 2.7.7.1) (Sorci
93 et al., 2007; Orsomando et al., 2012; Mori et al., 2014). All enzymes of this class, despite different
94 acronyms and EC classification that are somehow justified by enzyme-distinctive substrate
95 specificities, share common structure, catalytic mechanism, and essentiality for cell/organism
96 survival (Magni et al., 2004; Zhai et al., 2009; Pankiewicz et al., 2015). They thus represent very
97 promising druggable targets for developing novel drugs with either antibiotic or antiproliferative
98 activity (Magni et al., 2009; Petrelli et al., 2011). The findings of this work may provide new
99 insights into the industrial uses of Mexican sunflower as a renewable biomass to be exploited for
100 pharmaceutical applications.

101

102 **2. Materials and Methods**

103 *2.1. Plant material*

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

109 2.2. Hydrodistillation

110 Dry flowerheads (capitula) (350 g) were reduced into small pieces, then subjected to
111 hydrodistillation in a Clevenger-type apparatus for 4 h using 6 L of deionized water. The essential
112 oil yield (0.04%) was determined on a dry-weight basis (w/w). Once obtained, the oil was dried
113 (Na₂SO₄), stored into an amber glass flask, and kept at -20°C before chemical analysis and
114 biological experiments.

115 2.3. Chemicals

116 The analytical standards *n*-hexanal, *n*-nonane, α -pinene, camphene, benzaldehyde, β -pinene, 1-
117 octen-3-ol, *p*-cymene, limonene, γ -terpinene, terpinolene, linalool, *trans*-thujone, *trans*-pinocarveol,
118 terpinen-4-ol, α -terpineol, myrtenol, verbenone, *n*-decanal, geraniol, geranial, (*E*)-caryophyllene, α -
119 humulene, (*E*)- β -ionone, (*E*)-nerolidol, caryophyllene oxide, *n*-hexadecanoic acid were purchased
120 from SigmaAldrich (I-Milan) and used for identification of some peaks; (*E*)-Phytol was previously
121 isolated from *Onosma echioides* (Maggi et al., 2009). A mixture of *n*-alkanes (C₈-C₃₀) was
122 purchased from Supelco (Bellefonte, PA) and used to calculate the temperature-programmed
123 retention indices of chromatographic peaks. *n*-Hexane was purchased from Carlo Erba (I-Milan).

124 2.4. Chemical analysis of essential oil

125 Gas chromatographic separation of *T. diversifolia* of volatiles was achieved on an Agilent 6890N
126 gas chromatograph coupled to a 5973N mass spectrometer. For the purpose a HP-5 MS (5%
127 phenylmethylpolysiloxane, 30 m, 0.25 mm i.d., 0.1 μ m film thickness; J & W Scientific, Folsom)
128 capillary column was used with the following temperature programme: 5 min at 60°C, subsequently
129 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.

130 Injector and detector temperatures were 280°C. The carrier gas was He, with a flow rate of 1
131 mL/min. The split ratio employed was 1:50. Acquisition of mass spectra (m/z 29–400) was in
132 electron-impact (EI) mode with an ionization voltage of 70 eV. Before injection, the essential oil
133 was diluted 1:100 in *n*-hexane, then 2 μ L of the solution were injected into GC-MS system. For
134 identification of essential oil components co-injection with the above standards was used, together
135 with correspondence of retention indices and mass spectra with respect to literature data (Adams,
136 2007; NIST 08, 2008; FFNSC2, 2012). Semi-quantification of essential oil components was made
137 by peak area normalisation without calculating GC response factors.

138 2.5. Antibacterial activity

139 *T. diversifolia* essential oil was assayed by agar disc diffusion method against a panel of bacterial
140 species including *Staphylococcus aureus* ATCC 25923 (American Type Culture Collection,
141 Rockville, MD, USA), *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853,
142 *Enterococcus faecalis* ATCC 29212 following the Clinical and Laboratory Standards Institute
143 (CLSI) guidelines (CLSI, 2009). Strains were maintained overnight at 37 °C in blood agar plates
144 (Oxoid, Basingstoke, UK). Tested microorganisms ($1-2 \times 10^8$ cells per mL in saline) were spread on
145 the media plates using a sterile cotton swab. Paper discs of 6 mm in diameter were placed on the
146 surface of inoculated plates and spotted with 10 μ L of the essential oil. The plates were incubated
147 24 h at 35 ± 1 °C. The inhibition zones were measured with a calliper. A reading of more than 6
148 mm meant growth inhibition. The fluoroquinolone ciprofloxacin (5 μ g disc) was used as a reference
149 antibiotic. α -Pinene, the most prevalent compound into the essential oil, was also added to the
150 series to test its activity in the pure form (10 μ L per disc). The inhibition on *S. aureus* was also
151 investigated by the microdilution method following the international guidelines (CLSI, 2009).
152 Briefly, two-fold serial dilutions of mixture in Cation Adjusted Mueller Hinton Broth was set in 96-
153 well plates starting from 8 mg/l. An equal volume of the microbial inoculum (10^6 cfu/ml), obtained
154 by direct colony suspension of an overnight culture, was added to each well of the microtiter plate
155 containing 0.1 ml of the serially diluted test oil. After incubation for 18–24 h at 35 °C, in normal

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

159 2.6. Enzyme inhibition assay

160 Selected NaMN/NMN adenylyltransferase enzymes were chosen to represent either pathogenic
161 bacteria or their mammalian hosts. Pure recombinant NadD from *Staphylococcus aureus* subsp.
162 *aureus* N315 was a generous gift from Dr. Andrei Osterman (Sanford Burnham Prebys Medical
163 Discovery Institute, La Jolla, Ca, USA). The three *Mus musculus* isozymes NMNAT1, NMNAT2,
164 and NMNAT3 were obtained after bacterial overexpression and purification as previously described
165 (Orsomando et al., 2012). Enzyme rates were measured by using two equivalent spectrophotometric
166 coupled methods, namely a continuous assay based on detection at 340 nm of the NADH formed
167 (Balducci et al., 1995), and a discontinuous assay based on detection at 620 nm of the phosphate
168 formed (Vitali et al., 2015). The reaction mixtures, in addition to the buffer, substrates, and
169 ancillary system reactants indicated in Fig. 1 legend, contained 1.5-4 mU/mL of either *S. aureus*
170 NadD or one of the three mammalian NMNAT isoforms. The essential oil from *T. diversifolia* was
171 previously diluted in DMSO and then added to the reaction mixtures at 20-160 µg/mL final
172 concentration. Blank mixtures without the oil but with equal amounts of DMSO were set in parallel
173 and their rates fixed as 100% activity. Each enzyme was preincubated with the oil for 5 min at 37
174 °C, and then reactions were started by adding NMN for mammalian NMNATs or NaMN for
175 bacterial NadD. Measured rates were linear under these conditions for at least 20 min.

176 2.7. Antioxidant activity

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

182 power (FRAP assay) was carried out according to the procedure described by Müller et al. (2011),
183 by monitoring the reduction of Fe³⁺-tripyrindyl triazine (TPTZ) to blue-coloured Fe²⁺-TPTZ. The
184 ability of *T. diversifolia* essential oil to scavenge the different radicals in all assays was compared to
185 Trolox used as positive control and expressed as tocopherol-equivalent antioxidant capacity µmol
186 TE/g of product. Each experiment was repeated at least three times.

187 2.8. MTT assay

188 A375 (human malignant melanoma cells) and MDA-MB 231 cells (human breast adenocarcinoma
189 cells) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 2 mM L-glutamine,
190 100 IU/mL penicillin, 100 µg/mL streptomycin, and supplemented with 10% heat-inactivated fetal
191 bovine serum (HI-FBS). HCT116 cells (human colon carcinoma cells), were cultured in RPMI1640
192 medium with 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, and
193 supplemented with 10% HI-FBS. T98G cells (human glioblastoma multiforme cells) were cultured
194 in Eagle's minimum essential medium (EMEM) with 2 mM L-glutamine, 0.1 mM non-essential
195 amino acids, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 µg/mL streptomycin, and
196 supplemented with 10% HI-FBS. Cells were cultured in a humidified atmosphere at 37 °C in
197 presence of 5% CO₂. The MTT assay was used as a relative measure of cell viability. Cell-viability
198 assays were carried out as described (Quassinti et al., 2013). Briefly, cells were seeded at the
199 density of 2×10^4 cells/mL. After 24 h, samples were exposed to different concentrations of
200 essential oil (0.78-200 µg/mL). Cells were incubated for 72 h in a humidified atmosphere of 5%
201 CO₂ at 37 °C. Cisplatin was used as the positive control (0.05-20 µg/mL). At the end of incubation,
202 each well received 10 µL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide
203 (MTT) (5 mg/ml in phosphate-buffered saline, PBS) and the plates were incubated for 4 h at 37 °C.
204 The extent of MTT reduction was measured spectrophotometrically at 540 nm using a Titertek
205 Multiscan microElisa (Labsystems, FI-Helsinki). Experiments were conducted in triplicate.
206 Cytotoxicity is expressed as the concentration of compound inhibiting cell growth by 50% (IC₅₀).

207 The IC₅₀ values were determined with GraphPad Prism 4 computer program (GraphPad Software,
208 S. Diego, CA, USA).

209

210 **3. Results and Discussion**

211 *3.1. Essential oil analysis*

212 The composition of the essential oil distilled from flowerheads of *T. diversifolia* is reported in Table
213 1. The chemical profile, obtained by GC-MS, was very complex, with about 250 peaks detected. Of
214 these, 161 were identified, accounting for 87.3% of the total area. Eighteen volatile components
215 were present in relative percentages equal or above 1%, while the remaining (143 components) was
216 present in low relative percentages (below 1%). The oil was mainly composed of monoterpenes
217 (46.9%) with hydrocarbons and oxygenated compounds in similar amounts (24.7 and 22.2%,
218 respectively). Among them, α -pinene (13.7%), limonene (7.6%), and *cis*-chrysanthenol (6.2%) were
219 the most representatives. Sesquiterpenes (23.4%) were the second group characterizing the essential
220 oil, with oxygenated compounds more abundant than hydrocarbons (14.1 and 9.3%, respectively).
221 Main representatives of these classes were spathulenol (3.5%) and α -copaene (3.7%). Aliphatics
222 were a minor fraction of the oil (13.4%), with aldehydes (5.3%), saturated and unsaturated
223 hydrocarbons (3.5%), and fatty acids (3.1%) as the most abundant groups. They were mainly
224 represented by *n*-pentadecanal (1.6%), 1-pentadecene (1.7%) and *n*-hexadecanoic acid (3.0%),
225 respectively.

226 The composition herein reported showed some relevant differences with respect to previous works.
227 In particular, the essential oil examined was poor of (*Z*)- β -ocimene, which instead was reported as
228 one of the major volatile components of flowers (Menut et al., 1992). On the other hand, the sample
229 examined by us showed significantly lower levels of α -pinene with respect to samples from South
230 Africa and Cameroon previously analysed, where the relative percentages ranged from 61.0 to
231 72.8% (Lawal et al., 2012; Menut et al., 1992). Again, relevant differences in composition were
232 found with respect to sample from Nigeria (Moronkola et al., 2007), where the sesquiterpenes

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

240 3.2. Antimicrobial activity

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

254 3.3. Enzyme inhibition tests

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

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

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

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

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

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

285 Furthermore, on both inhibited enzyme targets, a dose dependent inhibitory effect was evidenced
286 (right panel), yielding to a similar IC₅₀ value of 60-70 µg/mL. Of note, all data in Fig. 1 refer to
287 assays carried out at substrates concentration just above the corresponding K_m, but very similar
288 results were also achieved using saturating concentration of both substrates (not shown), thus
289 suggesting a likely non competitive inhibition mechanism exerted by some oil component(s) yet to
290 be identified. Among the list presented in Table 1, most abundant are both α-pinene and limonene,
291 that were further tested individually against all enzymes above under similar assay conditions. As a
292 result, no inhibition was observed with both compounds (not shown), despite being tested at 20
293 µg/mL, *i.e.* a far higher concentration than predicted in the oil mixture.

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

299 Second, such potential bioactive components ~~drugs~~ contained in the *T. diversifolia* oil mixture
300 should be highly potent, having ruled out that the antibacterial effect is attributable the most
301 abundant α-pinene and limonene components of the mixture. We estimate, based on the relative
302 abundance of single compounds (see Table 1), that the observed inhibition is due to essential oil
303 components present in the mixture at sub-nanomolar concentrations. However, it is important to
304 underline that the low correlation between the MIC value obtained on *S. aureus* and SaNadD
305 inhibition is very likely due to a lack of cell permeability with this essential oil (Fericola et al.,
306 2015) or, otherwise, to specific bacterial defence mechanisms. Thus, further studies are in progress
307 in our group to improve the bacterial inhibition of the oil by combination with a cell membrane
308 penetration enhancer.

309 From a pharmacological point of view, the observed parallel targeting of NMNAT2 in the
310 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 IC₅₀ 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 IC₅₀ values of only 30 times lower than
322 that of trolox used as control for DPPH (108.8 $\mu\text{g}/\text{ml}$) and ABTS+ (41.7 $\mu\text{g}/\text{ml}$). 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 IC₅₀
345 values of 3.02, 3.79, and 3.46 µg/mL for A375, MDA-MB 231, and HCT116 cells, respectively.
346 These IC₅₀ values are close to those reported for cisplatin, an anticancer chemotherapy drug which
347 was used as the positive control (IC₅₀ 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 IC₅₀ 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 IC₅₀ 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, IC₅₀) (Jia et

363 al., 2013; Manuele et al., 2008), moreover it shows antitumor activity on lung adenocarcinoma
364 A549 (0.098 $\mu\text{L/mL}$, IC_{50}) and hepatocarcinoma HepG2 (0.150 $\mu\text{L/mL}$, IC_{50}) (Manassero et al.,
365 2013). Spathulenol was reported weakly active on human epidermoid carcinoma (KB) and inactive
366 on human breast cancer (BC) and human small cell lung cancer (NCIH187) cell lines (Prawat et al.
367 2013). To our knowledge, data reporting the cytotoxic activity of *cis*-chrysanthenol,
368 epoxyoctane, and α -copaene are missing. However, the concentrations of α -pinene (13.1%),
369 limonene (7.6%), and *cis* chrysanthenol (6.2%) cannot fully explain the cytotoxic activity of *T.*
370 *diversifolia* essential oil, which means that the other minor compounds contributed to the activity of
371 the essential oil or a synergism between the compounds increases their specific antiproliferative
372 activity.

373 In the plant screening program of the National Cancer Institute (NCI) of the USA, a crude oil is
374 generally considered promising as cytotoxic agent if the IC_{50} value, following incubation between
375 48 and 72 h, is less than 20 $\mu\text{g/mL}$ (Boik 2001). *T. diversifolia* essential oil showed an excellent
376 inhibitory activity against the three human tumor cell lines with IC_{50} values comparable to those of
377 the positive control, which in turn deserves further investigation. Worthy of mention was the
378 antiproliferative activity showed on glioblastoma that is particularly resistant to many
379 chemotherapies. The major drawbacks of chemotherapy for treatment of glioblastoma are the
380 incapability of many drugs to cross the blood-brain barrier. In this regard, the mexican sunflower
381 essential oil, being a mixture of many small lipophilic molecules, may represent a potential
382 treatment of these invasive tumors.

383

384 **4. Conclusions**

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

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

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415

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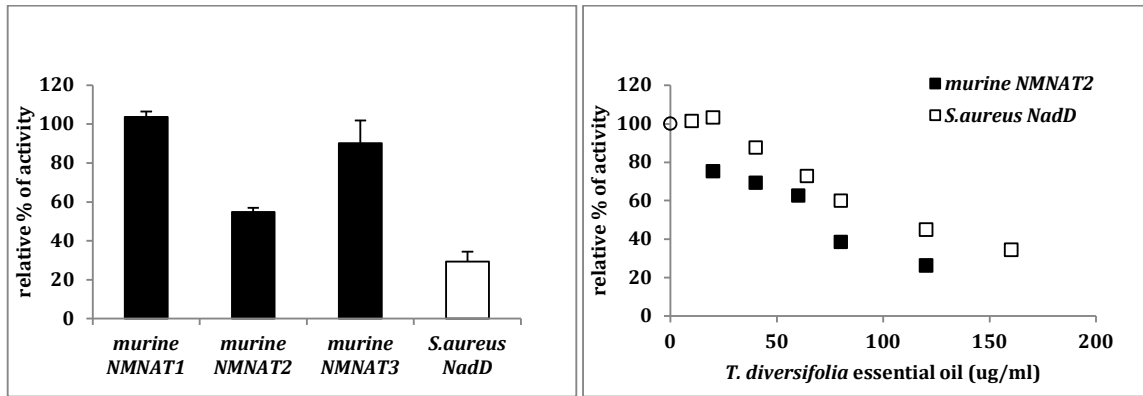


Figure 1. *In vitro* effect of *Tithonia diversifolia* essential oil on indispensable NaMN/NMN adenyltransferases from various sources. Data reported were from either the continuous (black bars and squares) or the discontinuous (white bar and squares) assay described in M&M, carried out at 80 µg/ml essential 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 MgCl₂, 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 MgCl₂, 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	<i>n</i> -hexanal	801	801	0,2	Std
2	1,3-cyclopentadiene, 1,2,5,5-tetramethyl-	837	835	Tr ^f	RI,MS
3	(2 <i>E</i>)-hexenal	850	846	Tr	RI,MS
4	<i>n</i> -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	<i>n</i> -nonane	899	900	0,1	Std
8	<i>n</i> -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 <i>E</i>)-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	<i>m</i> -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	<i>p</i> -cymene	1026	1024	0,4	Std
26	limonene	1030	1024	7,6	Std
27	(<i>Z</i>)- β -ocimene	1043	1032	0,3	RI,MS
28	benzene acetaldehyde	1048	1036	0,1	RI,MS
29	(<i>E</i>)- β -ocimene	1052	1044	0,2	RI,MS

30	γ -terpinene	1061	1054	0,3	Std
31	(2E)-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
	1,4-dimethyl-3-cyclohexenyl methyl				
50	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	<i>n</i> -decanal	1206	1201	0,5	Std
66	<i>trans</i> -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 <i>E</i>)-decen-1-ol	1258	1259	0,3	RI,MS
72	geraniol	1259	1249	0,3	Std
73	<i>n</i> -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 <i>E</i> ,4 <i>Z</i>)-decadienal	1294	1292	0,2	RI,MS
78	2-undecanone	1295	1293	0,1	RI,MS
79	<i>n</i> -tridecane	1300	1300	0,1	Std
80	2,3,4-trimethyl benzaldehyde	1311	1313	0,1	RI,MS
81	(2 <i>E</i> ,4 <i>E</i>)-decadienal	1316	1315	0,3	RI,MS
82	<i>p</i> -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	(<i>E</i>)- β -damascenone	1382	1383	0,1	RI,MS
90	β -isocomene	1393	1407	0,2	RI,MS

91	α - <i>cis</i> -bergamotene	1400	1411	0,1	RI,MS
92	<i>n</i> -tetradecane	1400	1400	0,1	Std
93	β -cedrene	1408	1419	0,2	RI,MS
94	(<i>E</i>)-caryophyllene	1408	1417	0,2	Std
95	α - <i>trans</i> -bergamotene	1432	1432	0,1	RI,MS
96	aromadendrene	1443	1439	0,1	RI,MS
97	α -humulene	1445	1454	0,1	Std
98	<i>allo</i> -aromadendrene	1452	1458	0,1	RI,MS
99	geranyl acetone	1454	1453	0,4	RI,MS
100	(<i>E</i>)- 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	(<i>E</i>)- 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	<i>epi</i> -cubebol	1488	1493	0,1	RI,MS
109	1-pentadecene	1493	1493	1,7	RI,MS
110	(<i>Z,E</i>)- 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	<i>trans</i> -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	(<i>E</i>)-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	<i>iso</i> -spathulenol	1630	1631*	0,2	RI,MS
128	<i>epi</i> - 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	<i>cis</i> -calamenen-10-ol	1653	1660	0,2	RI,MS
134	<i>trans</i> -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	<i>epi</i> -cyclocolorenone	1776	1774	0,1	RI,MS
144	1-tridecene-3,5,7,9,11-pentayne	1805	1809*	0,1	RI,MS
145	<i>n</i> -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 <i>E</i> ,9 <i>E</i>)-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	<i>n</i> -hexadecanoic acid	1965	1959	3,0	Std
152	methyl linoleate	2097	2095	0,1	RI,MS

153	<i>n</i> -heneicosane	2100	2100	0,1	Std
154	(<i>E</i>)-phytol	2116	2116	Tr	Std
155	<i>n</i> -tricosane	2300	2300	0,6	Std
156	<i>n</i> -tetracosane	2400	2400	0,1	Std
157	<i>n</i> -pentacosane	2500	2500	0,6	Std
158	methyl docosanoate	2534	2531	Tr	RI,MS
159	<i>n</i> -heptacosane	2700	2700	0,1	Std
160	squalene	2837	2847	0,1	RI,MS
161	<i>n</i> -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 C₈-C₃₀ 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 (\pm SD).

	<i>S. aureus</i>	<i>E. faecalis</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
	ATCC 29213	ATCC 29212	ATCC 25922	ATCC 27853
Essential oil	14.0 \pm 1.0	8.0 \pm 0.5	9.0 \pm 0.5	6.0 ^a
α -pinene	6.0 ^a	6.0 ^a	6.0 ^a	6.0 ^a
Ciprofloxacin	21.7 \pm 1.5	20.3 \pm 0.6	30.3 \pm 0.6	29.3 \pm 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	IC ₅₀	TEAC	IC ₅₀	TEAC
	μmolTE/g	μg/ml	μmolTE/g	μg/ml	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 IC₅₀ = 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.