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The effect of essential oil of *Syzygium cumini* on the development of granulomatous inflammation in mice

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Article

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Abstract: The anti-inflammatory and apoptotic activity of the essential oil of *Syzygium cumini* (L.) Skeels, Myrtaceae, leaves was investigated *in vivo*. The anti-inflammatory action and chronic granulomatous inflammation in BALB/c mice, intravenously infected with *Mycobacterium bovis*, BCG, (Bacillo Calmet Guerim), was judged by measuring and classifying the granulomas formed in the hepatic parenchyma. The degree of apoptosis in the inflammatory cells was also measured. A reduction in the granulomatous area and a change in the pattern of the granulomas were found. Anti-mycobacterial activity of the essential oil against *M. bovis* was detected *in vitro* by an interferometric method in liquid culture medium. The chemical constituents of the essential oil were determined by GC/MS. Higher yields of the essential oil of *S. cumini* leaves were obtained by extraction in a Clevenger apparatus when the fresh leaves were previously frozen as a pre-processing step. The essential oil obtained from this plant demonstrated a statistically significant and dramatic effect in the chosen model system.

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Introduction

The present work investigates the anti-inflammatory and anti-mycobacterial activity of the essential oil of *Syzygium cumini* (L.) Skeels, Myrtaceae, commonly known as jamum or jambul in Asia and as jambolão or jamelão in Brazil, is a tree from the Myrtaceae family, widely distributed in Asia and the Americas, and is known to have therapeutic properties, as can be seen in Table 1. Hypoglycemic activity has also been reported (Chirvan-Nia & Ratsimamanga, 1972; Achrekar et al., 1991; Pandey & Khan, 2002; Scharma et al, 2003; Stanely Mainzen Prince et al., 2003; Ayyanar & Subash-Babu, 2012).

Table 1. Previous uses of the plant parts of *S. cumini* in the treatment of various illnesses.

Illness	*S. cumini* parts			
	leaves	fruits	seeds	bark
diabetes	x	x	x	x
sores and ulcers			x	
dysentery		x	x	x
opium poisoning	x			
centipede bites	x			
gastric problems	x	x		x
repeated abortion				x
anorexia		x		x
headache		x		x
renal problems	x			

Source: Ayyanar & Subash-Babu, 2012.

There is evidence of antimicrobial and anti-inflammatory action of the extracts, applicable in both acute and chronic granulomatous inflammations (Muruganandan et al., 2001; 2002; Modi et al., 2010). Pro-apoptotic properties in leukocytes were also reported by Ling-Ling et al. (2000). The main components in extracts from leaves from the Myrtaceae family that are obtained by means of hydroalcoholic extraction are flavonoids and phenolics (Slowing et al., 1994; Mahmoud et al., 2001). These are known to have anti-inflammatory properties (Brasseur, 1989). On the other hand, it is known that the essential oil, which does not contain non-volatile flavonoids, also have anti-inflammatory action (Siani et al., 2000). The essential oil of *S. cumini* obtained from leaves is mainly composed of mono and sesquiterpenes, principally, α-pinene and β-caryophyllene (Siani et al., 2000; Ayyanar & Subash-Babu, 2012).

One of the main problems in diseases of allergic or parasitic origin and in bacterial lung inflammation is the excessive presence of eosinophils or neutrophils in the locus of inflammation. Tissue damage has been attributed to high concentrations of these leukocytes. The high concentration of mononuclear leukocytes, neutrophils and eosinophils in inflammatory reactions induced by lipopolysaccharides (LPS) are inhibited by *S. cumini* oil treatment (Siani et al., 2000). On the other hand, in an inflammatory reaction induced by chest injected BCG, which was characterized by a mononuclear and polymorphonuclear cell profile 24 h after infection, the *S. cumini* essential oil provoked an enhancement of mononuclear leukocytes and eosinophils (Menezes-de-Lima Jr et al., 1997). Menezes-de-Lima Jr. also found that the essential oil completely inhibited the production of cytokines and nitric oxide *in vitro*.

The anti-inflammatory action of the principal components of the essential oil in an isolated form was also tested by experiments where inflammation in mice was induced by pleura injected LPS. Treatment with the monoterpene α-pinene was not able to inhibit migration of eosinophils but application of the sesquiterpene β-caryophyllene did reduce the migration significantly (Ocete et. al., 1989; Martin et al., 1993).

The present work is concerned with possible influences of the essential oil of *S. cumini* with regard to the inflammatory processes caused by Mycobacterium bovis infection. The well known pattern of inflammation caused by *M. bovis* can be used as a model to study granulomatous inflammation caused by tuberculosis (Aarestrup et al., 1995). This illness is one of the predominant causes of human mortality from infectious agents, with a death rate of 1.5 million individuals per year (WHO, 2011). The experimental model used in the present work is the classical one that uses BCG infected mice BALB/c (Raja, 2004). In the present study, the anti-inflammatory, apoptotic and anti-mycobacterial action of the essential oil extracted from leaves of *S. cumini* was investigated. We also identified an improved method for the isolation of the essential oil and characterized its composition by Gas Chromatography-Mass Spectrometry (GC/MS).

Materials and Methods

Essential oil of Syzygium cumini

Fresh leaves of *Syzygium cumini* (L.) Skeels, Myrtaceae, were collected and separated into three parts. One part was dried at ambient temperature in air, a second part was stored in plastic Ziploc® bags and frozen at -20 °C and the third was used in fresh form for essential oil extraction. The essential oil of *S. cumin*i was extracted from dried, frozen and fresh leaves by hydrodistillation using a Clevenger apparatus for 4 h. The oil used in the biological assays was not exposed to any solvents to separate the oil from the water. However, the oil used in the analysis of its constituents was separated from the water with the help of ether, as describe below.

The leaves were collected in March of 2001 on the campus of the Federal University of Juiz de Fora located in the South-Western region of Brazil (S21°46'46.4" W43°22'13.3" elevation 865 m). The plant material was identified and authenticated by Dr. Fátima Regina Gonçalves Salimena (Leopoldo Krieger Herbarium at the Federal University of Juiz de Fora) and voucher specimen is deposited as exsiccate CESJ46601. The oil thus obtained was stored in amber glass vials and was stored in a freezer at -20 °C. The oil employed in the experiments with BCG had been stored for approximately 6 to 12 months after extraction.

Analysis of the essential oil

The hydrodistillate of freshly collected and frozen leaves, obtained as previously described, was extracted with 3x 10 mL portions of previously distilled diethyl ether. The pooled ether extracts were dried over Na_2SO_4, filtered and evaporated using a rotary film evaporator, T≤30 °C, until a final volume of approximately 0.5 mL. The clear solution thus obtained was transferred directly to a 1.5 mL GC/MS vial. The evaporation flask was rinsed additionally twice with 0.4 mL of ether and the solvent evaporated under a slow, steady stream of dry N_2. All glassware employed was cleaned by soaking in a solution of KOH in isopropyl alcohol, followed by rinsing with distilled water until pH≤7, followed by ethanol and finally, a small aliquot of distilled ether. The procedure was performed in duplicate and, in accordance with Good Laboratory Practices, included a method blank.

The essential oil thus obtained was diluted with 1 mL of distilled ether and analyzed on a gas chromatograph

coupled to a mass spectrometer detector (GCMS-QP2010 Plus; Shimadzu). Samples were injected using an automated injector (AOC-5000), in the split mode (1:10). The column employed was a Restek Rtx-5MS®, (5% diphenyl bound to polysiloxane, 30 m long, 0.25 mm I.D.). The rate of temperature change and other parameters employed were as follows: initial oven temperature: 50 °C was maintained for 5 min, and then raised at the rate of 4 °C/min until 200 °C. This temperature was maintained for a further 5 min. to expulse any semi-volatile components present. The total run time was 47.50 min. The temperature of the ion source was 200 °C, the GC/MS interface was kept at 220 °C, and the cut time before beginning GC/MS detection was 5 min. The mass spectra were acquired at 70 eV and were scanned between 40-500 AMU.

Data treatment

Due to the lack of pure reference standards for all of the constituents identified by the NIST 9.0 library furnished with the GC/MS employed in the analysis, the following criteria were employed in the data treatment. A correlation above 90% with the NIST library was arbitrarily chosen as a "positive" identification. No attempt was made to correct for response factors. The total ion current and area under the peak are assumed to correspond approximately to the % composition of each component due to their structural similarity. As such, the data reported are qualitative and semi-quantitative at best. Components which constituted less than 1% of the total ion current are not reported.

In vivo *and* in vitro *tests to study the action of essential oil on BCG inflammatory pattern and BCG growth*

The *in vivo* investigations were performed in a way that is compatible with the current principle of "3Rs" (NC3Rs, 2012) of the Animal Ethics Committee (Ministério da Saúde, 2004). The animals used in the experiment were furnished by the Center of Reproductive Biology, UFJF-MG, Brazil in 2001 and the experiments were approved by this institution. The experiments obeyed Brazilian Federal Law no 6.638, May 8th, 1979, which was the legislation in 2001. A total of 24 BALB/c strain female mice, with an average weight of 22 g were intravenously infected by injecting $2x10^6$ CFU (colony-forming units) of BCG strain Pasteur. All animals were kept under identical conditions with respect to food, water, temperature, and luminosity. Groups of six animals were used per test group: two control groups C21, C28 and two treated groups T21 and T28 that were sacrificed 21 and 28 days after infection respectively. These periods of time were chosen considering that 21 days is the typical period to develop abundant immature granulomas, and 28 days after infection corresponding to the apex of mature granulomas when epithelioid cells predominate (Aarestrup et al., 1995; 2000). The treated groups received 300 µL of essential oil per kg of body weight every day with gavage applications of 0.1 mL sterile Phosphate Buffered Saline (PBS) solution (concentration 66 mL oil/1L solution). The oil was administered as an emulsion (oil + PBS, vigorously shaken) that was prepared freshly every day. The treatment started on the day of infection and continued until the animals were sacrificed. The concentration employed (66 ppm v/v) was based on previously obtained positive results using the extract of S. cumini (Muruganandan et al. 2001). The animals of the control groups received gavage applications of the same volume of sterile PBS in order to eliminate any possible matrix effects of the PBS vehicle. In order to test toxicity of the essential oil assays with brine shrimp *Artemia salina* were performed according to McLaughlin (1998). Despite the fact that LC50 (Letal Concentration 50%) was found to be between 0.1 µL/mL and 0.2 µL/mL none of the animals of the treated groups T21 and T28 died before sacrifice.

The livers of the sacrificed animals were collected and fixed in calcium buffered formalin, embedded in paraffin, and sectioned into 5 µm thick slices. The histopathologic analyses were carried out with three slices per animal, two of which (separated by 50 µm) were used for classifying granulomas in up to 100 microscopic fields per slice (magnification 400x). The third served to measure granulomatous area and to judge apoptotic processes in the hepatic parenchyma and in the granulomas (magnification 400x using the Image Pro Plus® program). The slices for histopathologic examination and classification of granulomas were stained using the standard hematoxilin-eosin technique and the apoptotic samples were prepared according to the TUNEL method (TdT FragEL-DNA Fragmentation Detection kit, Boston, MA, 02118-2518, USA).

The analysis of apoptosis was performed counting the cases of apoptosis of inflammatory cells within a granuloma and measuring the area of the granuloma so as to obtain the ratio α=number of apoptosis/area of granuloma, which is expected to be roughly proportional to the fraction of apoptosis and the total number of leukocytes of the granuloma. This ratio, α, was determined for a large number of granulomas in such a way that the error of the mean value for every animal becomes smaller than the variation from animal to animal. The error of the mean value for each test group was then determined statistically based on the number of animals rather than by the total number of granulomas. The effect of the essential oil on the development of the granulomatous inflammation was tested with three observable quantities using a 2-tailed Mann-Whitney test with 95% confidence interval as described below.

The state of inflammation was judged by measuring the total area occupied by granulomas in the field. The corresponding results are represented as fraction

of granulomatous area and the area of the microscopic field. The granulomas were also classified according to the following criteria: Ex= exudative; corresponding to a mixed and non-organized concentration of leukocytes with the appearance of an exudate but which differs form a pure exudate by the presence of some adjacent macrophages (data not shown), M=mixed; a geometrically well shaped cluster of epithelioid cells and lymphocytes with random localization of these species (Figure 1A), O=organized; a geometrically well shaped cluster of epithelioid cells and lymphocytes where the lymphocytes form a rim (Figure 1B), Mφ = Macrophagic granuloma; a well shaped cluster that contains predominantly macrophages (including epithelioid cells) (Figure 1C), and F = Fibrotic granuloma with some collagenous fibers (data not shown).

Tests of anti-mycobacterial action of the essential oil were performed *in vitro* employing the interferometric method (Jardim et al., 2003), where bacterial activity is monitored by measuring the change in refractive index of the nutrient solution caused by bacterial metabolism. Five experiments were performed with (2.5±0.1) mL of Bacto Middlebrook 7H9 culture media enriched with oleic acid albumin dextrose catalase (OADC) in the interferometer. All five experiments used 4 x 10^6 CFU of BCG. The experiments BCGa) BCGb) contained no essential oil and in the remaining three experiments, essential oil was mixed with the samples in the following concentrations: SCa (2.4±0.2) x10^{-3} µL oil/mL solution, and experiments SCb and SCc (0.24±0.02) µL oil/mL solution. In order to compare our results with a conventional antibiotic, interferometric measurements were also performed with samples containing rifampicin (Lot 780773, SIGMA) with concentrations zero (control), and 4 and 250 ng/mL (positive control). This experiment used smaller sample holders with a smaller stock of oxygen. The changes in refractive index were monitored continuously. At the beginning of the experiments, the same specimen of BCG was also seeded in 7H10 agar culture medium enriched with OADC in order to have a conventional control of bacterial viability. Furthermore, at the end of each experiment the content of the sample holder was seeded to observe whether there existed any contamination. All experiments were found to be free of contamination. At the beginning of experiment SCb conventional experiments were also conducted to evaluate bacterial growth in the presence of the essential oil: four test tubes with liquid culture medium and BCG were prepared with 4x10^6 CFU of BCG each. In two of the test tubes, essential oil was added to achieve a final concentration of (0.24±0.02) µL/mL. The four tubes were incubated at 37 °C and monitored regularly by visual inspection. Tests with solid culture medium 7H10 enriched with OADC in Petri dishes of 5 cm diameter were also performed. Seven dishes were inoculated with 4x10^6 CFU of BCG. All dishes had a central disc of sterile paper with 10 mm diameter. Five paper discs were impregnated with 1.2 µL *S. cumini* oil and the dishes were held at 37 °C.

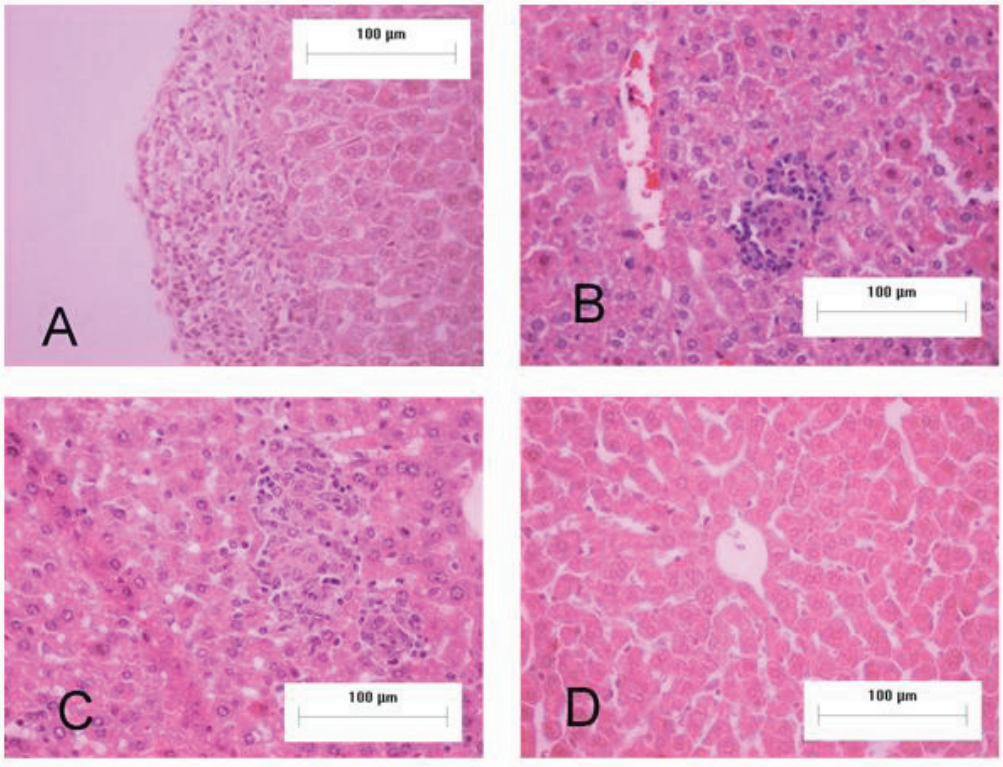

Figure 1. A. Example of a mixed granuloma (M); B. Example of an organized granuloma (O); C. Example of a macrophagic granuloma (Mφ); D. Example of a normal hepatic parenchyma (magnification 400x).

Results

Enhancement of essential oil yield

Upon extraction of the essential oil from fresh or dried leaves of *S. cumini*, we obtained very low yields (less than 10 μL/kg). After freezing the fresh leaves prior to extraction, it was possible to increase the yield to 0.5 mL/kg. We suspect that the freeze-thaw cycle ruptures vesicles or other plant structures which contain the steam volatile components and that this method may also be useful for other plants with low essential oil yields.

Analysis of principal chemical constituents

We have identified and confirmed, with a high degree of certainty, the principal constituents of the essential oil of *S. cumini* used for this study. The majority of the constituents are sesquiterpenes of the carane family, namely α-caryophyllene and β-caryophyllene, its alcohol and epoxide as well as terpineol (Figure 2). Table 2 summarizes the constituents we were able to identify, their retention times (R_t), relative % composition based on peak area, and the % correlation to the NIST 9.0 library.

Table 2. Principal components of the essential oil of fresh *Syzygium cumini* leaves, their retention times on GC-MS chromatography and relative percent composition.

RT	Area %	Substance
14.360	1.33	*trans*-β-ocimene
20.649	9.08	α-terpineol
24.226	1.99	bornyl acetate
29.068	25.24	α-caryophyllene
29.412	2.00	carveol
30.173	16.00	β-caryophyllene
30.643	1.19	α-muurolene
32.377	4.90	*iso*-caryophyllene
33.892	3.90	caryophyllenyl alcohol
34.306	3.83	caryophyllene oxide
34.830	4.82	β-humulene
36.362	5.23	epiglobulol

Histopathologic analysis

In all four test groups the hepatic parenchyma showed interstitial edema with perivascular granulomas composed of T-cells and epithelioid cells. However, a normal pattern of hepatic parenchyma was also observed in different regions (Figure 1D). The polymorphonuclear cell content was limited to very small numbers of cells (typically 1 to 7 cells) although roughly 30% of the granulomas did have these cells. No statistically significant difference in the apoptotic process among the four groups was found (see Table 3). In the group C28, numerous macrophagic granulomas were found. As described in the previous section, the granulomas were classified in order to judge their patterns. Figures 1A, B, C show examples of the categories M, O, and Mφ. No granulomas with predominant polymorphonuclear leukocyte populations were observed, however, the presence of such cells was also detected.

Table 3. Comparison of mean values of percentage of granulomatous area, number of apoptosis per area of granuloma and type ratio Mφ/M for the control and treated groups corresponding to 21 and 28 days after infection.

Test group	%GA	α [μm^{-2}]	Mφ/M
C21	1.3±0.3	(1.9±0.4) x 10^{-3}	0.33±0.07
T21	1.0±0.2	(1.9±0.5) x 10^{-3}	0.6±0.1
C28	5.6±1.9*	(1.3±0.4) x 10^{-3}**	1.4±0.4***
T28	1.3±0.4*	(2.7±0.5) x 10^{-3}**	0.4±0.1***

*p-value 0.026; **p-value 0.082; ***p-value 0.009.

Figure 3 shows the relative frequencies of the types of granulomas. As can be seen, the relative frequency of the organized type is practically the same in all groups. On the other hand, the categories M and Mφ show an interesting behavior. Therefore, it would seem to be appropriate to define the quotient of relative frequencies $\tilde{M\varphi}/M$ (number of Macrophagic granulomas divided by the number of mixed granulomas) as a relevant parameter. In this fashion, the morphology of the granulomas can be characterized using a single parameter. Table 3 shows the mean values of the following characteristic quantities: %GA=percentage of granulomatous area in a field; α=number of apoptosis per granulomatous area, and $\tilde{M\varphi}/M$.

The groups sacrificed 21 days after infection do not show any difference between treated and control animals. However, the control experiment shows a significant growth of granulomatous area in the time interval from 21 to 28 days, where, for the treated animals, %AG remained approximately constant during that time interval. The comparison of the groups C28 and T28 shows significant differences in the granulomatous area %AG and the type ratio $\tilde{M\varphi}/M$. This indicates that essential oil treatment was able to dramatically inhibit the growth of granulomatous areas which occurs during the fourth week post infection in non-treated animals.

Analysis of antimycobacterial action

Figures 4A and B show the negative time derivative of the refractive index of the nutrient solution of the five interferometric experiments. This quantity, *-dn/dt*, is a measure for the rate of consumption of nutrients and

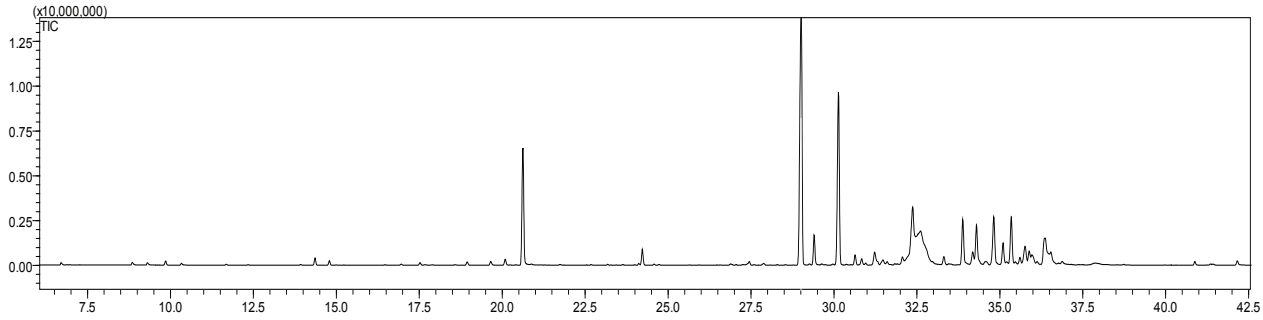

Figure 2. A typical gas chromatogram of the essential oil from the fresh leaves of *Syzygium cumini.*

can be correlated to the state of bacterial activity. As can be seen from Figure 4A, the experiments with 0.24 µL/mL of *S. cumini* oil have no, or a very low bacterial activity and no bacterial growth is visible. The curves without S. cumini oil and the one with the low concentration (0.0024 µL/mL) show a pronounced bacterial activity and bacterial growth. The interferometric assays show that a concentration of 0.24 µL/mL of essential oil has an antibiotic action similar to 0.25 µg/mL of rifampicin (Figure 4B). The conventional experiment in liquid culture medium showed abundant colonies after seventeen days in the test tubes without S. cumini oil and no visible colonies in the tubes with 0.24 µL/mL of *S. cumini* oil. After 21 days, the control Petri dishes without oil were densely occupied with colonies visible to the unaided eye and the dishes with oil had a halo that occupied almost the entire dish (Figure 5).

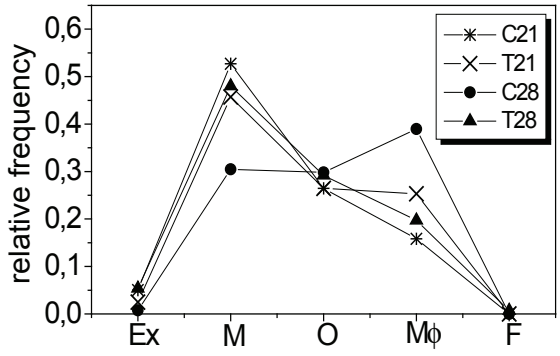

Figure 3. Mean relative frequencies of granuloma types for the four test groups: star: C21; cross: T21; full circle: C28 and full triangle: T28. The type pattern of the groups C21, T21 and T28 are approximately the same and the profile of the group C28 is significantly different, revealing an enhancement of Macrophagic granulomas (see the quotient Mφ/M in Table 3). Granuloma type classification: Ex= exudative; M=mixed; O=organized; Mφ= Macrophagic; F = Fibrotic. The graph serves to define and illustrate the relevant parameter Mφ/M, which reveals a difference in the inflammatory development with 95% confidence interval according to a 2-tailed Mann-Whitney test (compare Table 3).

Discussion

The results obtained in the present work demonstrate conclusively that the essential oil of *Syzygium cumini* (L.) Skeels, Myrtaceae, has an antimicrobial action capable of stopping the proliferation of *Mycobacterium bovis in vitro* and that the development of granulomatous inflammation caused by BCG in the hepatic parenchyma of mice is modified after 21 days of infection. The dramatic increase of granulomatous area that occurs in the time interval (21 days, 28 days) in non-treated animals is not observed in the treated group. The increase of granulomatous area in the control group during the fourth week after infection can be explained assuming that an exponential growth of bacteria occurs and bacteria are subsequently released. Thereafter, resident activated macrophages and/or recruited monocytes from the bloodstream phagocytize these bacilli, which results in the formation of predominantly macrophagic granulomas (Harrison et al., 2005). This explains the observed increase of the parameter Mφ/M. The release of bacteria from macrophages may be due to lysis of macrophages caused by internal multiplication of the bacteria (Harrison et al., 2005) or by apoptosis of macrophages (Raja, 2004). Although the apoptosis of infected macrophages is a mechanism of host defense, this mechanism is not sufficiently efficient to guarantee that all bacteria contained in the dying macrophage are killed (Condos et al., 1997; Tan et al., 1997). The net effect of apoptosis is an inhibition of bacterial growth (Oddo et al., 1998; Keane et al., 2000) and the main contribution to the growth of granulomatous areas during the fourth week after infection is mostly likely due to lysis.

The principal constituents identified in the essential oil of *S. cumini* are caryophyllene isomers, α-terpineol, and oxygenated derivitaves of caryophyllene (alcohol and epoxide). These are ubiquitous in the plant kingdom and their biological activity has been studied extensively. The α-caryophyllene and the β-caryophyllene are known for their anti-inflammatory potential (Fernandes et al., 2007; Gertsch et al., 2008). The anti-inflammatory

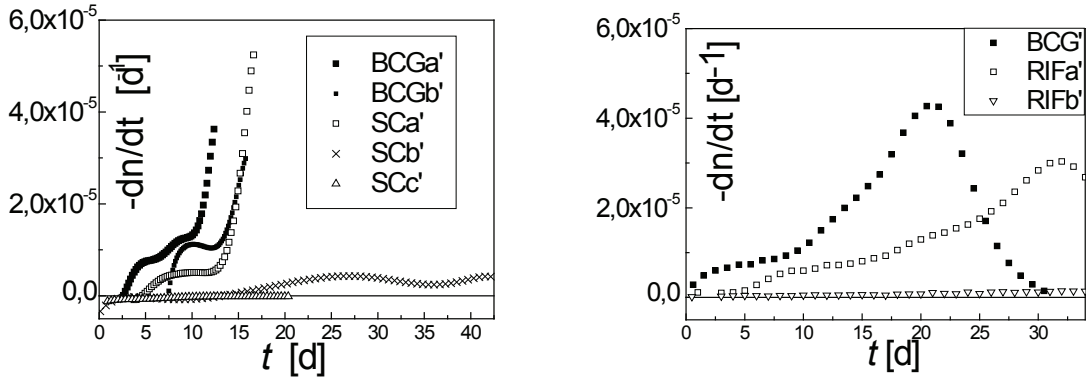

Figure 4. Negative time derivative of refractive index of the liquid culture medium as a function of time with BCG containing samples. A. Full squares: BCGa' and BCGb'=BCG+culture medium (no essential oil); open squares: SCa'=BCG+culture medium+essential oil with concentration 0.0024 µL/mL; crosses: SCb'=BCG+culture medium+essential oil with concentration 0.24 µL/mL; open triangles: SCc'=BCG+culture medium+essential oil with concentration 0.24 µL/mL. The curves corresponing to the concentration 0.24 µL/mL show no or very low bacterial activity and no bacterial exponential growth. B. full squares: BCG'=BCG+culture medium (no rifampicin); open squares: RIFa'=BCG+culture medium+rifampicin with concentration 0.004 µg/mL; open triangles: RIFb'=BCG+culture medium+rifampicin with concentration 0.25 µg/mL. The sudden decrease in the curves BCG' and RIFa' is due to oxygen depletion.

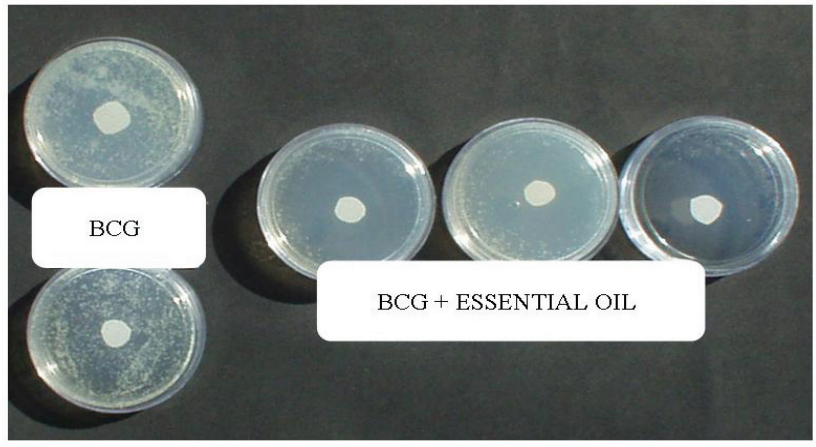

Figure 5. Petri dishes with 7H10+OADC (enrichment) seeded with 4 x 10^6 CFU of BCG with central discs of sterile paper, three of which were wetted with *S. cumini* essential oil. The picture shows the state 21 days after seeding without and with *Syzygium cumini* essential oil. The two dishes to the left show abundant colonies whereas the dishes with essential oil show a halo the covers almost the entire dish.

effects of α-caryophyllene were shown to be comparable with dexamethasone (Fernandes et al. 2007). The possible mechanisms of action of β-caryophyllene have been studied already. β-caryophyllene is recognized as a CB_2 receptor agonist (Gertsch et al., 2008; Bento et al., 2011), while its oxide has proven to be cytotoxic (Neung et al., 2011).

According to Galiègue et al. (1995), CB_2 receptors are present in the principal immune defense cells such as polymorphonuclear neutrophil cells, T-lymphocytes and macrophages that are involved in chronic inflammation investigated in the present work. Moreover, Gertsch et al. (2008) described β-caryophyllene as a specific CB_2 receptor agonist. Our results indicate that the activation of immune cells by β-caryophyllene may have an anti-inflammatory effect, which is in accordance with observations in carrageenan-induced inflammation (Gertsch et al., 2008). The exact mechanism of action in the present model system is not completely understood, but may be mediated by CB_2 receptors or by the cytotoxic effect of one or more of its components. The interferometric experiments demonstrated the antimycobacterial action of the *S. cumini* essential oil *in vitro*. Therefore, one might surmise that the presence of the oil inhibits the bacterial growth in macrophages and the liberation of bacilli through lysis is stopped. The

average number of apoptosis per area of granuloma (α) in the group T28 is about two times larger than in the group C28. The statistic analysis does not permit the conclusion that the oil treatment increases the α-value. Nevertheless, it does show that the presence of the essential oil did not decrease apoptosis. In this manner, the apoptotic host defense mechanism is maintained in the presence of the oil.

Conclusions

The assays permit us to conclude that the essential oil of *S. cumini* show relevant anti-inflammatory activity *in vivo* and antimycobacterial action *in vitro*. The analyses of the essential oil composition revealed major quantities of caryophyllenes and its isomers and oxygenated derivatives. As a possible explanation, based on the literature, we suggest that the high percentage of β-caryophyllene identified in the essential oil is likely to be responsible for the anti-inflammatory activity. The antimycobacterial action observed *in vitro* may be related to the cytotoxic effect present in the caryophyllene oxide also detected in the oil. The mechanism of action present in anti-inflammatory effect of α-caryophyllene has to be better investigated. A method to enhance the yield of essential oil of *S. cumini* by freezing leaves prior to hydro-distillation was discovered. Thus, the study of the essential oil of *S. cumini* showed that this plant deserves further investigation of its biological activities.

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Authors contributions

RRPM colleted leaves of *S. cumini*, extracted the essential oil, infected, treated and sacrificed the mice, processed histological slides and performed histopathologic analyses. RRPM also performed antimicrobial sensibility tests including the interferometric ones, participated in statistical analysis and manuscript edition. DFJ developed and applied the interferometric measurements. ARS participated in the animal experiments. ES and RLF collected plant samples for exsiccate preparation and performed toxicity tests of the essential oil. AGC and RMG performed the chromatographic analysis of the essential oil. RMG suggested mechanisms of action of the oil and revised the manuscript. JPRFM participated in the development of the interferometric method. BL designed and developed the interferometric method advising DFJ. He also participated in the classification of granulomas, statistical analysis, performed interferometric antimicrobial sensibility tests and participated in manuscript edition. FMA designed the project to study the effect of the essential oil in the granulomatous chronic inflammation advising RRPM to perform the animal assays. All the authors have read the final manuscript and approved the submission.

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