Evaluation of *in vitro* antileishmanial and antimycobacterial activities of *Stifftia chrysantha* J.C. Mikan extracts

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Resumo

Stifftia chrysantha J.C. Mikan é uma planta pertencente à família Asteraceae cujo principal uso pela população é o ornamental e atualmente se encontra sob risco moderado de extinção. É sabido que a planta foi utilizada no tratamento de afecções respiratórias por quilombolas. O objetivo deste estudo foi investigar o potencial efeito antimicrobiano de diferentes extratos de *S. chrysantha* contra algumas espécies de micobactérias e formas promastigotas de duas espécies de *Leishmania*. Os testes foram realizados *in vitro* utilizando MTT ou Resazurina em métodos colorimétricos, de acordo com o microrganismo avaliado. Os resultados mostraram baixa atividade dos extratos contra as culturas de micobactérias. Por outro lado, um efeito inibidor do crescimento foi observado no extrato metanólico das folhas e no extrato hexânico da casca contra as culturas de promastigotas de *L. amazonensis* (CI_{50} = 55,16 μg/mL extrato metanólico and 38,61 μg/mL extrato hexânico) and *L. chagasi* (CI_{50} = 72,05 μg/mL extrato hexânico). Novos estudos são necessários para descobrir as substâncias responsáveis pela inibição do crescimento das formas promastigotas.

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Abstract

Stifftia chrysantha J.C. Mikan is a plant that belongs to Asteraceae family, mainly used for ornamental purposes and it is moderately endangered to die out nowadays. It is known this plant has been used on the treatment of respiratory affections by quilombo communities (Brazilian hinterland settlement founded by people of African origin). The aim of this study was to investigate the potential antimicrobial effect of different extracts from *S. chrysantha* against some species of mycobacterias and promastigote forms of two *Leishmania* sp. *In vitro* assays were performed using colorimetric methods with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide or Resazurin, according to the microorganism evaluated. The results showed low activity of extracts against mycobacterial cultures. On the other hand, a growth inhibitory effect was observed in the methanol extract from leaves and on the hexane extract from bark against promastigote culture of *L. amazonensis* (IC_{50} = 55.16 μg/mL methanol extract and 38.61μg/mL hexane extract) and *L. chagasi* (IC_{50} = 72.05 μg/mL hexane extract). New studies are necessary to discover the substances that were responsible for the growth inhibition of promastigote forms.

Introduction

Stifftia chrysantha J.C. Mikan (Asteraceae) is popularly known as diadem, fox tail or gold rain due to the color and shape of its inflorescences. Brazilian popular names of the plant are: rabo-de-cotia, diadema, pompom, flor-da-amizade, esponja, esponja-de-ouro, jambeiro do-mato, pincel. Its main use by the population is ornamental and it is moderately endangered to die out nowadays, according to list annexed to the Decree 19.149 published by the Rio de Janeiro Environmental Protection Bureau (2000). It can be found in Protected Areas such as the National Park of Tijuca, Rio de Janeiro Botanical Garden and Grajaú-Jacarepaguá Road, in Rio de Janeiro, RJ, Brazil (Crespo et al., 2010). Few studies on the chemical composition of this plant have been reported. Oliveira (1999) described flavonoids isolated from its flowers while Marques (2012) found a significant predominance of methyl salicylate in the volatile fractions from fruits ranging from 85% to 95% in the volatile mixture as well as such presence in all aerial parts of the plant during all seasons of the year.

The organic ester methyl salicylate seems to take part in the attraction process of pollinators and in the defense of the plant. Besides, the metabolic conversion of methyl salicylate into salicylic acid and subsequently into acetyl salicylic could justify the ancient use of the plant by quilombo communities for treatment of flu, colds and respiratory affections (Marques et al., 2005). This metabolic conversion from methyl salicylate to salicylic acid is also important for the plant's defense system and for signaling against predators' attacks. Salicylic acid is known as an important phytoalexin present in responses to physical and biological stresses suffered by the plant (Durrant et al., 2004). Employing a rapid radiometric method, Lall et al. (1999) detected a significant inhibition of *Mycobacterium tuberculosis* H37Rv exposing the bacteria to the extracts of *Polygala myrtifolia* (Polygalaceae), a plant that shows high concentration of methyl salicylate. The reported activity found in *P. myrtifolia* and the similar major compound content present in the aerial parts of *Stifftia chrysantha* motivated the investigation about the biological potential this native Brazilian endangered risk species *S. chrysantha* extracts and also to the pure methyl salicylate against mycobacteria. We also tested the activity of the extracts against promastigote forms of leishmania. The diseases caused by mycobacteria and *Leishmania sp*. represent important disorders for Public Health, since long times are needed for effective treatment by using drugs that may cause potential side effects (Almeida et al., 2005; Medeiros et al., 2005; Coll et al., 2009; Sundar et al., 2007). Many research works have been performed with the attempt to identify new therapeutically potential drugs against tuberculosis, mycobacteriosis and leishmaniasis. Such studies have been characterized by the use of preliminary approaches with *in vitro* experiments, before *in vivo* ones and clinical trials (Lahlou et al., 2004).

Material and Methods

Reagents: Dimethyl-sulfoxide (DMSO); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and Methyl salicylate were purchased from Sigma Chemical Co, St. Louis, MO, USA); Resazurin sodium salt powder was purchased from Acros Organic N.V., Geel, Belgium).

Collection of aerial parts and roots of S. chrysantha: Aerial parts and roots of *S. chrysantha* (leaves, flowers, barks, branches and fruits) were collected under supervision of botanist Roberto L. Esteves in the garden of National Museum of Rio de Janeiro in December 2004. The voucher number is R208153. The plant material was collected early in the morning from the same chosen specimen and it was taken immediately to the laboratory and separately reduced into small pieces. The powdered materials were air-dried.

Preparation of S. chrysantha extracts: Air-dried and powdered plant materials (50g of leaf; 30g of bark and 40g of flower) were separately extracted under static maceration using hexane as solvent, and followed by methanol. Removal of residual solvent under reduced pressure was performed using a Büchi rotatory evaporator, equipped with warm bath under controlled temperature (40oC). The same procedure was carried out with methanol as solvent extractor.

Extracts used: hexane extract from leaves and bark of *S. chrysantha*; and methanol extract from leaves and fruits of *S. chrysantha*.

Methyl salicylate: methyl salicylate was used in the experiments on mycobacteria at the following concentrations: 250μg/mL, 125μg/mL, 63μg/mL, 31μg/mL, 16μg/mL and 8μg/mL and using DMSO as diluent.

***Leishmania* sp. assays:** promastigote forms of *Leishmania amazonensis* (MHOM/Br75/Josefa) isolated from a patient who had diffuse cutaneous form of leishmaniasis) were cultured in Warren medium (BHI, plus hemin and folic acid) and promastigote forms of *L. chagasi* (MHOM/Br/74/PP75 isolated from patients who had visceral form of leishmaniasis) were cultured in 199 medium, both supplemented with fetal bovine serum and maintained at 24ºC during one week. Fetal bovine serum (FBS) was purchased from Cultilab (Campinas, São Paulo, Brazil). Brain heart infusion (BHI) from Himédia (Mumbai, Indian). Hemin, folic acid, and 199 medium were purchased from Sigma Chemical Co (St. Louis, MO, USA). Biological activity against promastigote forms of *L. amazonensis* and *L. chagasi* was determined using the MTT colorimetric method based on reduction of the salt by mitochondrial dehydrogenases (Mossman et al., 1983). Promastigoste forms in logarithmic growth stage of *in vitro* growth of both species were used in the experiments. They were added to flat-bottomed 96-well plastic tissue-culture plates at the concentrations 2.0 x 106cells/mL and 3.0 x 10^6cells/mL of *L. amazonensis* and *L. chagasi*, respectively. After 1h and at 24ºC, the parasites were exposed to different concentrations of extracts of *S. chrysantha* previously solubilized in DMSO. Concentrations (C) of the extracts were 250.0μg/mL, 125.0μg/mL, 62.5μg/mL, 31.3μg/mL, 15.6μg/mL and 7.8μg/mL and each concentration were performed in triplicate and in two independent assays. The promastigote forms were exposed to the extracts, to Amphotericin B (standard drug used as the positive control) and to the DMSO solution at 0.01% for 72h 24ºC. The colorimetry was assessed by absorbance using SPECTRAMAX 190, Molecular Devices spectrometer and 570nm filter. For the results analysis, *GraFit* (Erithacus Software Ltd., Horley, U.K) software version 5 was used.

Experiments using rapidly and slow growing mycobacteria: the susceptibility tests for mycobacteria using extracts were performed in 96-well plates using the colorimetric test based on resazurin reduction following the procedure used by Palomino et al. (2002). All experiments were performed in triplicates and at least three repetitions. Concentrated solutions (5mg/mL) of the extracts were prepared by initially solubilizing the extracts in DMSO and subsequently in sterile water. The total content of DMSO in each well reached 10%, which did not inhibit mycobacterial growth. In flat-bottomed 96-well plastic tissue-culture plates, dilutions of the extract were prepared in Middlebrook 7H9 broth (BD-lote.2112134-USA) culture medium enriched with OADC (Becton-Dikinson) at the following concentrations: 2500μg/mL; 1250μg/mL; 625μg/mL; 313μg/mL; 156μg/mL and 78μg/mL resulting in a final volume of 100μL in each well. Next, 100μL of slow-growing mycobacteria (*M. tuberculosis* H37Rv – ATCC 27294, M. bovis – BCG (Monroe) or rapidly growing mycobacteria (*M. smegmatis* – ATCC 14468, *M. abscessus* – ATCC 199777, *M. chelonae* – ATCC 5752) suspension was added to the extract solutions separately. The final concentrations of the extracts into the wells were: 1250μg/mL; 625μg/mL; 313μg/mL; 156μg/mL; 78μg/mL and 39μg/mL. The suspensions were prepared in Middlebrook 7H9 (Difco) culture medium enriched with OADC (Becton-Dikinson) at 1:25 concentration from the initial suspension and turbidity equivalent to 1.0 in McFarland scale. Standard drugs were used as positive control for inhibition of mycobacteria growth, such as rifampicin (3-(4-methylpiperazinyliminomethyl)- rifamycin – Lot 780773 – SIGMA) against slow-growing mycobacteria (*M. tuberculosis* and *M. bovis*) and ciprofloxacin (ALDRICH Chemistry – Lot: 17850) against rapidly growing mycobacteria (*M. smegmatis*, *M. abscessus* and *M. chelonae*) at the following concentrations: rifampicin – 32 – 0.3 X 10^{-6}μg/mL and ciprofloxacin – 0.5 – 0.9 x 10^{-7}μg/mL, considering a serial dilution (1:2) for both drugs. The plates were sealed and kept at 37ºC for 7 days. On the seventh day, in sterile environment, 10μL of resazurin 0.01% (diluted in etylenglicol and sterile distilled water) was

added (Resazurin Sodium Salt Powder Acros Organic N.V., Geel, Belgium). Extracts were considered as active against mycobacteria when exhibited MIC < 200μg/mL (Tosun et al., 2004).

Results and Discussion

The experiments performed in order to evaluate potential antimycobacterial activity of extracts from different parts of *S. chrysantha* did not show biological activity against any of the mycobacteria tested. This judgment is based on a criterion established by Tosun et al. (2004), which considers a substance as active if its MIC < 200μg/mL. However this limit is controversial. Lima (2006) evaluated the antimicrobial activity of extracts obtained from different Brazilian plant species and verified antimycobacterial activity of methanol extracts from leaves of *Lafoensia pacari* against *M. smegmatis* (MIC = 1250μg/mL), M. fortuitum (MIC = 1250μg/mL) and M. phlei (MIC = 625μg/mL) not considering hence, the activity limits determined by Tosun et al. (2004).

In our study, two MIC values were determined: one against *M. smegmatis* which resulted from the methanol extract from leaves (1125.1 ± 0.13μg/mL) and another against *M. chelonae* (312.5 ± 0.16μg/mL), related to methanol extract from flowers, as shown in Table 1. These found MIC values may indicate the presence of substances capable of interfering with the metabolism of *M. chelonae* and *M. smegmatis*. The other evaluated plant products did not show antimycobacterial activity even at higher concentration. It still has to be discovered the active compounds in the *S. chrysantha* extracts. It is known that the *S. chrysantha* extracts contain considerable amounts of quercetin an important flavonoid (Oliveira, 1999). As it is known, several functions can be attributed to the flavonoids, such as protection of the plants against insects, fungal and bacterial colonization, viral infections as well as attraction of pollinators due to the remarkable colors of these compounds (Salvador et al., 2008). Boligon (2012) studied antimycobacterial activity of quercetin against slowly and rapidly growing mycobacteria and they encountered a MIC > 200μg/mL that might indicate that quercetin is one of the active compounds of the *S. chrysantha* extract.

Seasonal evaluation of *S. chrysantha* volatile fractions revealed methyl salicylate as the major constituent of flowers, fruits and leaves. The high content of this compound was observed mailing in the fruits and flowers during the whole year, suggesting an importance in the attraction process of pollinators, defense system and/or for signaling against predators' attacks (Marques et al., 2012). Methyl salicylate has already been related to antimycobacterial activity by Lall and co-workers (1999) that verified a significant effect of *Polygala myrtifolia* (Polygalaceae) extract, which contains great quantities of methyl salicylate. In addition, methyl salicylate (83.8%, 89.1% and 97.8%) was found as the main volatile constituent in roots of the *P. sabulosa*, *P. paniculata* and *P. cyparissia*, respectively (Pizzolati et al. 2009). Therefore, one might surmise that this compound could be responsible for antimycobacterial properties.

In our study, antimycobacterial activity of methyl salicylate has been evaluated for the first time and the results revealed no inhibitory activity on the mycobacterial growth (Table 1). The results suggests that the significant inhibition of *M. tuberculosis* H37Rv found by Lall (1999), is not due to the exclusive action of methyl salicylate, the major compounds of that extract. This preliminary screening against *Mycobacterium tuberculosis*, H37Rv was performed using acetone and water plant extracts. The minimal inhibitory concentration of *Polygala myrtifolia* was 0.1 mg/ml, being also active against the resistant strain at 0.1 mg/ml. Despite of the good activity displayed by *P. myrtifolia* extract, no phytochemical separation was performed in the related study. A chemical investigation of the genus *Polygala* showed the occurrence of a variety of secondary metabolites, such as xanthones, saponins, oligosaccharides, flavonoids, coumarins and styrylpyrones (Johannl et al. 2011). Some of these compounds are enable to be extracted by the acetone and/or water preparations and could be acting against the *M. tuberculosis* studied strain.

The hexane extract from the bark was active against both *Leishmania* species (*L. amazonensis*: IC50 = 38.61 ± 0.48μg/mL; *L. chagasi*: IC50 = 72.05 ± 4.28μg/mL), while the methanol extract from the leaves was active only against *L. amazonensis* (IC50 = 55.16 ± 5.08μg/mL). The other extracts did not reveal inhibitory activity of promastigote forms growth, at least in the range of experiments performed.

Table 1: Inhibitory effect of *Stifftia chrysantha* extracts and methyl salicylate against *Mycobacterium and Leishmania* species of medical interest.

EVALUATED PRODUCT	*M. smegmatis* MIC (μg/mL)	*M. chelonae* MIC (μg/mL)	*M. abscessus* MIC (μg/mL)	*M. tuberculosis* MIC (μg/mL)	*M. bovis* MIC (μg/mL)	*L. amazonensis* IC_{50} (μg/mL)	*L. chagasi* IC_{50} (μg/mL)
Fruit (MeOH)	N.I	N.I.	N.I.	N.I.	N.I.	N.I.	N.I.
Flower (MeOH)	N.I.	**312.5 ± 0.16**	N.I.	N.I.	N.I.	N.I.	N.I.
Leaves (MeOH)	**1125.1 ± 0.13**	N.I.	N.I.	N.I.	N.I.	**55.16 ± 5.08**	N.I.
Bark (Hex)	N.I.	N.I.	N.I.	N.I.	N.I.	**38.61 ± 0.48**	**72.05 ± 4.28**
Leaves (Hex)	N.I.	N.I.	N.I.	N.I.	N.I.	N.I.	N.I.
Methyl salicylate	N.I.	N.I.	N.I.	N.I.	N.I.	N. E.	N. E.
Ciprofloxacin	0.018 ± 0.012	0.014 ± 0.015	0.21 ± 0.26	---	---	---	---
Amphotericin B	---	---	---	---	---	0.9 ± 0.0001	1.9 ± 0.0001
Rifampicin	---	---	---	0.818 ± 1.280	0.004 ± 0.001	---	---

In vitro Antimycobaterial and antileishmanial experiments using extracts of *S. chrysantha*, methyl salicylate and standard antimicrobials.
N.I.: No inhibition; N.E.: Not evaluated.

Guided by the considerations concerning about antimycobacterial activity one may guess that the leishmanicidal activity might also be related to the quercetin. This hypothesis is supported by findings in the literature. For example, Sarkar and colleagues (2002) detected reduction of leishmanias in the spleen of hamsters of this phytocompound. This reduction is probably due to an interference of quercetin in the leishmania's iron metabolism (Sen et al., 2008).

In conclusion, the biological activities verified from the extracts from different parts of *S. chrysantha* aggregate a relevant value to the plant, once it was the first time that the antimycobacterial and antileishmanial potential activity has been evaluated. Despite the low activity found against mycobacteria culture, the promastigote culture suffered a significant inhibition of its growth. However, new studies are necessary in order to elucidate which phytocompounds in the plant material are responsible for the biological activity and how they act against the *Leishmania* species exposed to the products used in this study.

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