

Benedito Rodrigues da Silva Neto  
(Organizador)

# Pesquisa Científica e Tecnológica em Microbiologia 3



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## APRESENTAÇÃO

Temos o prazer de dar continuidade ao tema de microbiologia inter-relacionado à pesquisa científica e tecnológica iniciado pela editora no ano de 2019. Apresentamos aqui um novo volume deste contexto, denominado “Pesquisa científica e tecnológica em microbiologia, volume 3” contendo trabalhos e pesquisas desenvolvidas em diversos institutos do território nacional contendo análises de processos biológicos embasados em células microbianas ou estudos científicos na fundamentação de atividades microbianas com capacidade de interferir nos processos de saúde/doença.

A microbiologia é um vasto campo que inclui o estudo dos seres vivos microscópicos nos seus mais variados aspectos como morfologia, estrutura, fisiologia, reprodução, genética, taxonomia, interação com outros organismos e com o ambiente além de aplicações biotecnológicas. Como uma ciência básica a microbiologia utiliza células microbianas para analisar os processos fundamentais da vida, e como ciência aplicada ela é praticamente a linha de frente de avanços importantes na medicina, agricultura e na indústria.

A microbiologia como ciência iniciou a cerca de 200 anos, entretanto os avanços na área molecular como a descoberta do DNA elevou a um novo nível os estudos desses seres microscópicos, além de abrir novas frentes de pesquisa e estudo. Sabemos na atualidade que os microrganismos são encontrados em praticamente todos os lugares, e a falta de conhecimento que havia antes da invenção do microscópio hoje não é mais um problema no estudo, principalmente das enfermidades relacionadas aos agentes como bactérias, vírus, fungos e protozoários.

Temas ligados à pesquisa e tecnologia microbiana são, deste modo, discutidos aqui com a proposta de fundamentar o conhecimento de acadêmicos, mestres e todos aqueles que de alguma forma se interessam pela saúde em seus aspectos microbiológicos. Portanto a obra propõe uma teoria bem fundamentada nos resultados práticos obtidos em alguns campos da microbiologia, abrindo perspectivas futuras para os demais pesquisadores de outras subáreas da microbiologia.

Assim, desejo a todos uma ótima leitura!

Benedito Rodrigues da Silva Neto



## SUMÁRIO

<b>CAPÍTULO 1</b> .....	<b>1</b>
ANÁLISE FITOQUÍMICA E ATIVIDADE ANTIBACTERIANA DE EXTRATOS DA CASCA DOS FRUTOS DE <i>Hymenaea courbaril</i> L SOBRE <i>Staphylococcus aureus</i>	
Diogo Siebra Alencar Gleilton Weyne Passos Sales Suelen Carneiro de Medeiros Mary Anne Medeiros Bandeira Nádia Accioly Pinto Nogueira	
<b>DOI 10.22533/at.ed.4352001071</b>	
<b>CAPÍTULO 2</b> .....	<b>12</b>
ATIVIDADE ANTIBACTERIANA DE EXTRATOS DE FOLHAS E CASCA DE <i>Jacaratia spinosa</i> (Aubli) A. DC. (MAMOEIRO-BRAVO)	
Katiele Pelegrini João Augusto Firmino de Carvalho Jakson José Ferreira Graciele Fernanda de Souza Pinto	
<b>DOI 10.22533/at.ed.4352001072</b>	
<b>CAPÍTULO 3</b> .....	<b>18</b>
AVALIAÇÃO DA TOXICIDADE AGUDA E DA CITOTOXICIDADE DOS EXTRATOS ETANÓLICOS DA MACRÓFITA <i>Hydrocotyle bonariensis</i> Lam (APIACEAE)	
Andreza Larissa do Nascimento Joyce Bezerra Guedes Antônia Ângela Bezerra José Fabricio de Carvalho Leal Maria do Socorro Meireles de Deus Ana Paula Peron Márcia Maria Mendes Marques Duque Ana Carolina Landim Pacheco	
<b>DOI 10.22533/at.ed.4352001073</b>	
<b>CAPÍTULO 4</b> .....	<b>35</b>
O ESTADO DA ARTE DO COMPLEXO <i>Cryptococcus neoformans</i> E DA CRIPTOCOCOSE	
Lucas Daniel Quinteiro de Oliveira Lúcia Kioko Hasimoto e Souza Benedito Rodrigues da Silva Neto	
<b>DOI 10.22533/at.ed.4352001074</b>	
<b>CAPÍTULO 5</b> .....	<b>57</b>
<i>Corynebacterium pseudotuberculosis</i> PROTEIN EXTRACT INDUCES IP10 PRODUCTION IN BLOOD SAMPLES OF INDIVIDUALS WITH PULMONARY TUBERCULOSIS	
Rogério Reis Conceição Samanta Queiroz dos Santos Zunara Victória Santana Batista Ramon Mendes dos Santos Silvânia Maria Andrade Cerqueira Caio Lopes Borges Andrade Soraya Castro Trindade Fúlvia Soares Campos de Sousa Lília Ferreira de Moura-Costa Marcos Borges Ribeiro	

Roberto Meyer  
Songelí Menezes Freire  
DOI 10.22533/at.ed.4352001075

**CAPÍTULO 6 ..... 66**

EFFECTS OF SUB-INHIBITORY CONCENTRATION OF ANTIMICROBIALS IN *Bacteroides fragilis* STRAINS ISOLATED FROM INTRA-ABDOMINAL INFECTIONS

Marcela Abreu Menezes  
Priscila Simão Costa  
João Paulo Amaral Haddad  
Cristina Dutra Vieira  
Luiz de Macêdo Farias  
Simone Gonçalves dos Santos

DOI 10.22533/at.ed.4352001076

**CAPÍTULO 7 ..... 83**

EFICÁCIA DE ÁLCOOL GEL COMO ANTIMICROBIANO DE SUPERFÍCIES INERTES

Cristiane Coimbra de Paula  
Fabrício Caram Vieira  
João Pedro Castoldo Passos  
Caroline Aquino Vieira de Lamare  
Walkiria Shimoya-Bittencourt

DOI 10.22533/at.ed.4352001077

**CAPÍTULO 8 ..... 91**

EVALUACIÓN DE GENES DE RESISTENCIA A ANTIMICROBIANOS EN BACTERIAS DEL TRACTO GASTROINTESTINAL DE NOVILLOS ALIMENTADOS CON EXTRACTO DE ORÉGANO

Maria Juliana Moncada Diaz  
Luciano Antônio Ritt  
Michele Bertoni Mann  
Ana Paula Guedes Frazzon  
Jeverson Frazzon  
Vivian Fischer

DOI 10.22533/at.ed.4352001078

**CAPÍTULO 9 ..... 100**

OBTENÇÃO DE CELULASES MICROBIANAS: UMA BREVE REVISÃO

Tatielle Pereira Silva  
Alexsandra Nascimento Ferreira  
Cledson Barros de Souza  
Dávida Maria Ribeiro Cardoso dos Santos  
Marta Maria Oliveira dos Santos  
Hugo Juarez Vieira Pereira

DOI 10.22533/at.ed.4352001079

**SOBRE O ORGANIZADOR..... 111**

**ÍNDICE REMISSIVO ..... 112**

# CAPÍTULO 5

## *Corynebacterium pseudotuberculosis* PROTEIN EXTRACT INDUCES IP10 PRODUCTION IN BLOOD SAMPLES OF INDIVIDUALS WITH PULMONARY TUBERCULOSIS

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**ABSTRACT:** *Mycobacterium tuberculosis* and *Corynebacterium pseudotuberculosis* are part



of the CMRN group and share similarities among some virulence/pathogenicity factors. The objective of the present study was to use semi-purified extracts of *C. pseudotuberculosis* strains to evaluate antigenicity in samples of individuals diagnosed with active pulmonary tuberculosis. Voluntary participants with positive tuberculosis (TB) diagnosis and treatment-naïve tuberculosis (n=28), without complaints, no history or symptomatology of TB or *M. tuberculosis* infection (n=28). With heparinized blood, cell stimulation was carried out using mitogen (PWM) and *C. pseudotuberculosis* protein extract (PAT-10, VD57 and FRC-41 strains). For cytokines measurement, BD Cytometric Bead Array (CBA) was used. Semi-purified extracts of all three *C. pseudotuberculosis* strains induced production of cytokines in blood samples from studied groups. In addition, they differentiated studied groups when compared to IFN- $\gamma$  and IP-10. *C. pseudotuberculosis* strains extracts PAT10, VD57 and FRC41 induced production of IFN- $\gamma$ , IP-10 in blood samples from individuals infected with *M. tuberculosis*. Under stimuli of extracts used, the detection of IFN- $\gamma$  and IP-10 differentiated the control group and active TB groups.

**KEYWORDS:** *Corynebacterium Pseudotuberculosis*; Cytokines; *Mycobacterium Tuberculosis*; Tuberculosis; Antigenicity

## 1 | INTRODUCTION

Several scientific researches are carried out to identify and obtain reagents that make up laboratory diagnostic kits, and for that, molecules (antigens) of the infectious agents are necessary for evaluation of the host response with *in vivo* or *in vitro* models. Some biomolecules can be shared by different biological agents, such as *Mycobacterium tuberculosis* (*M. tuberculosis*) and *Corynebacterium pseudotuberculosis*, which can be used in cross-reactive studies, to evaluate immune response and diagnostic tests <sup>[1]</sup> and it's cost benefit. The diagnosis of *M. tuberculosis* infection should be made clinically and by screening methods. When suggestive, it should be confirmed by laboratory methods, with agent identification, such as in sputum microscopy and molecular tests <sup>[2]</sup>. In a country with a high incidence rate, such as Brazil, despite the current technological resources used to control tuberculosis, it could be considered necessary to seek innovative strategies regarding diagnostic methods and immunoprophylaxis, aimed at the early diagnosis of TB cases, and its effective treatment, since rapid and safe diagnosis, accompanied by early treatment, are fundamental measures to break the transmission chain and control tuberculosis <sup>[3]</sup>. On the other hand, the infection by *C. pseudotuberculosis* that compromises superficial and deep lymph nodes has a course of infection that may involve respiratory organs in goats and sheep presents an infectious model similar to that of human tuberculosis <sup>[4]</sup>. Additionally, due to the continuously discussed similarities between some virulence / pathogenicity factors of these bacterias, the objective of this study was to use semi-purified extracts of *C. pseudotuberculosis* strains to investigate their *in vitro* antigenic

capacity, in the cellular immune response by cytokines release as a model proposed with samples from individuals with active pulmonary tuberculosis.

## 2 | MATERIALS AND METHODS

### 2.1 Ethical aspects and Selection of Study Participants

This study was approved by the Research Ethics Committee of the Institute of Health Sciences - CEP-ICS / UFBA (CAAE nº 57662016.8.1001.5662). The study population consisted of 56 volunteers from the Bahia lung disease specialized Hospital and a clinical laboratory in a public University of Bahia through march-june 2017 categorized into two groups. Group 1 (control, n = 28): Individuals without complaints, history or symptoms of pulmonary TB and without M. tuberculosis infection (negative Tuberculin Skin Test / non-reactive TST) and with normal white blood cell count (WBC). Group 2 (active pulmonary TB, n = 28): Individuals with TB diagnosis, as recommended by the Ministry of Health protocols performed at the service unit, (Positive Molecular Rapid Test (GeneXpert®) without resistance to rifampicin, positive smear microscopy and / or culture of BK positive) and with normal WBC. Samples from this group were collected before beginning treatment. Samples of participants who reported contact or consumption of derivatives (meat and / or raw milk) of small ruminants were not included in the analysis avoiding research vies by cross reaction.

### 2.2 Production of extracts of *Corynebacterium pseudotuberculosis*

Three strains of *C. pseudotuberculosis* were used: strain PAT10, of a pulmonary abscess in a sheep from Argentina Patagonia <sup>[5]</sup>, strain VD57 isolated from a granulomatous lesion in a goat of the municipality of Juazeiro, State of Bahia <sup>[6]</sup> and strain FRC41, isolated from the inguinal lymph node of a 12-year-old girl with necrotizing lymphadenitis in France <sup>[7]</sup>. The cultures in Brain Heart Infusion (BHI), the three-phasepartitioning (TPP) technique was performed, according to <sup>[8]</sup>, with minor modifications. Protein dosage was performed using Bio-Rad Protein Assay (Lowry).

### 2.3 Blood collection

Samples of blood were obtained into heparinized tube to perform the *in vitro* cell stimuli and in EDTA tube for WBC Counting.

### 2.4 *In vitro* stimulation with *Corynebacterium pseudotuberculosis*

In a sterile 24-well plate for cell culture, 0.5 ml of blood per well was added to each extract at a protein concentration of 25ug / ml, as established in previous standardization.

The pokeweed (PWM) was employed at a concentration of 5ug / ml as a positive control. Pure blood and stimulus samples were incubated at 37°C for 24 hours. The supernatants obtained by centrifugation were immediately stored at -20°C for subsequent cytokine dosing.

## 2.5 Dosing of cytokines

The cytokines IFN- $\gamma$  dosage were performed by the Human Th1 / Th2 / Th17 Cytokine Kit (BD®) and IP-10 was dosed by the Human IP-10 CBA Flex Set Kit (BD®), both following the manufacturer's recommendations. Acquisition was predetermined for 3,000 events in FACSCalibur-BD, with Cell Quest Pro Software (BD®). Analysis were done in the FCAP Array™ software.

## 2.6 Statistical analysis

For the data normality analysis, the Kolmogorov-Smirnov test was used. T-test was used to analyze cytokine data with parametric (normal) distribution and Mann-Whitney test for nonparametric (abnormal) distribution. Values of  $p < 0.05$  were considered statistically significant. Data obtained in the study were analyzed in GraphPadPrism v 5.0 software.

## 3 | RESULTS

Epidemiological characteristics of the studied population are described in table 1. It is observed that female participants are in a higher proportion than the male participants in the control group, unlike the TB group in which there is an equivalent proportion of both sexes.

	Variable	Control (N=28)	TB (N=28)
<b>Sex</b>	Female	20 (71,5%)	14 (50,0%)
	Male	8 (28,5%)	14 (50,0%)
<b>Age</b>	Mean (Interval)	35 (20 – 63)	36 (19 – 55)
<b>Observation of BCG scar</b>	Yes	24	22
	No	4	6
<b>TB contact</b>	Domicile	3	7
	Work	4	8
	Other	1	5
	No Contact	20	8

Table 1 - Clinical-epidemiological characteristics of the study population.

Figures 1 and 2 show the median concentrations of IFN- $\gamma$  and IP-10 cytokines in the whole blood samples after in vitro stimulation in the two study groups.

In the control group, the median concentrations of IFN- $\gamma$  without stimulus (S / EST), basal condition, were zero and the value of minimum and maximum of 0.0 - 20.61. There



was statistical significance in the intragroup comparison with the stimuli PAT10, VD57, FRC41 ( $p < 0.0001$ ). In the TB group, the median IFN- $\gamma$  S / EST concentrations were 4.18 pg / mL and the minimum and maximum values of 0.0-31.88, in the intragroup comparison, significance was obtained in the stimuli VD57 ( $p = 0.0114$ ) and FRC41 ( $p = 0.0062$ ). In both groups the medians of the cytokine dosed under all stimuli were larger than the basal. In the intergroup analysis there was significance between S / EST ( $p = 0.0045$ ), PAT10 ( $p = 0.0050$ ) and VD57 ( $p = 0.0007$ ). There is a lower concentration of IFN- $\gamma$  dosed in the different stimuli of the TB group, in relation to the control group, except in basal condition (Figure 1).

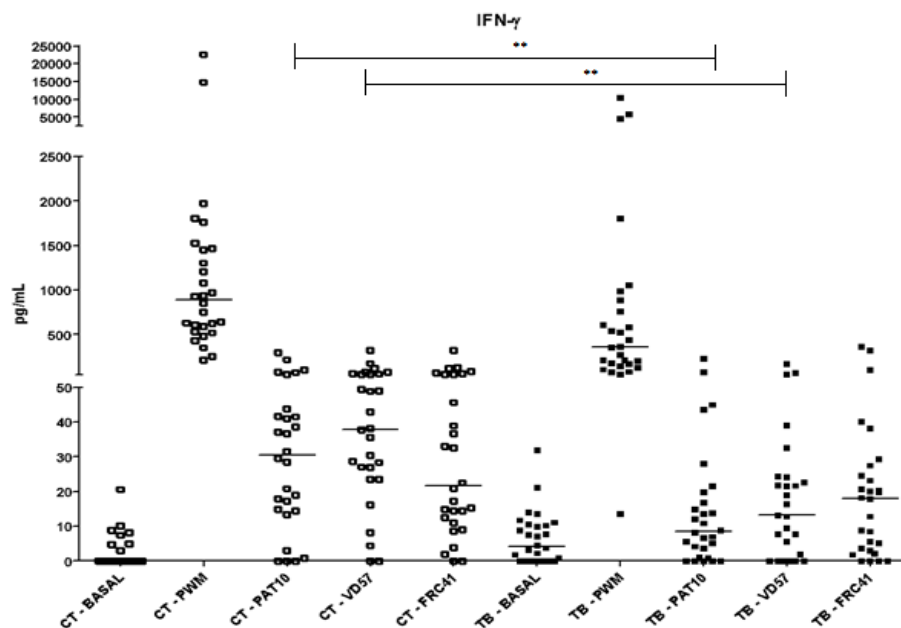


Figure 1 - IFN- $\gamma$  in peripheral blood stimulated in vitro for 24 hours with extracts antigen secreted by *C. pseudotuberculosis* strains. The median value is expressed in the horizontal line. Being: Control / CT ( $n = 28$ ) and Tuberculosis / TB ( $n = 28$ ). PWM: pokeweed; PAT10, VD57, FRC41: extracts of the strains PAT10, VD57 and FRC41 of *C. pseudotuberculosis*, respectively. Pre-determined acquisition of 3000 events with the Cytokine-BD Human Th1 / Th2 / Th17 Kit using Cell Quest Pro-BD® Software. Analysis software: FCAP Array BD®. Statistical analysis: In-group: Basal X CT-PAT10, CT-VD57, CT-FRC41 (all  $p < 0.0001$ ), BASAL X TB-VD57 ( $p = 0.0114$ ), X TB-FRC41).

The median concentrations and the minimum and maximum values of IP-10 in the control group were 252.2pg / mL and 26.11 - 2806, respectively. The median IP-10 concentrations of basal blood in the control group were lower than the median values under stimulus, PAT10, VD57, FRC41 ( $p < 0.0001$ ). In the TB group, basal median was 877.9pg / mL and minimum and maximum values were 101.6 - 7799. IP-10 concentrations were higher under stimulation with PAT10 ( $p = 0.0044$ ), VD57 ( $p < 0.0001$ ) and FRC41 ( $p = 0.0032$ ) than from basal. The IP-10 concentrations of the TB group were higher in relation to the control group. There is a difference of the IP-10 dosed between the CTxTB groups in the conditions BASAL ( $p < 0.0001$ ), PAT10 ( $p = 0.0470$ ) and FRC41 ( $p = 0.0044$ ) (Figure 2)

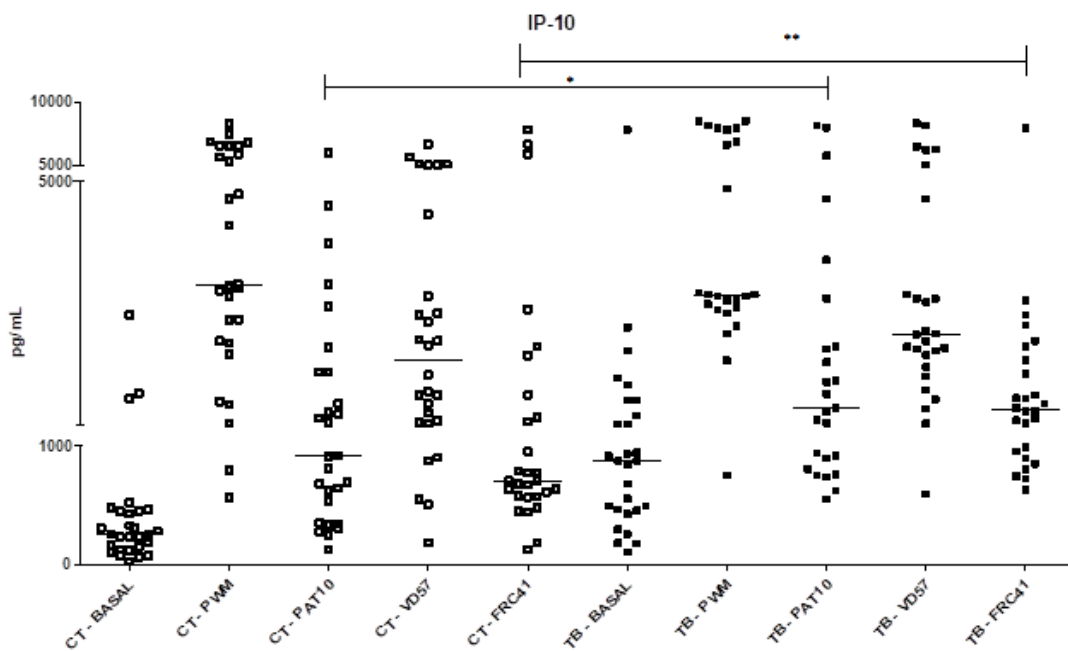


Figure 2 -IP-10 in peripheral blood stimulated for 24 hours with antigenic extract secreted by *C. pseudotuberculosis* strains. The median value is expressed in the horizontal line. Being: Control / CT (n = 28) and Tuberculosis / TB (n = 28). PWM: pokeweed; PAT10, VD57, FRC41: extracts of the strains PAT10, VD57 and FRC41 of *C. pseudotuberculosis*, respectively. Pre-determined acquisition of 3000 events with the BD™ CBA Human IP-10 Flex kit using Cell Quest Pro-BD® software. Analysis software: FCAP Array BD®. CT-PAT10, CT-VD57, CT-FRC41 (p <0.0001); TB-VD57 (p <0.0001) TB-FRC41 (p = 0.0032). The continued bar presents statistical analysis of the difference between groups (CTxTB).

#### 4 | DISCUSSION

The profile of the effective immune response against *M. tuberculosis* is Th1, IFN- $\gamma$  is the main cytokine involved in this context, but the action of the cytokine alone is not sufficient to promote protection against this bacillus, and thus the contribution of components cell and other cytokines are required to promote effective protection, such as TNF, IL-2 and IL-6 [9]; [10]. Chemokines such as IP-10 also aid the Th1 response profile. On the other hand, the development of Th2 profile involves IL-4 and other cytokines, which act to inhibit the Th1 response against *M. tuberculosis*. IL-10 exerts functions similar to IL-4 [9]; [11]. The antigenic potential of the *C. pseudotuberculosis* semi-purified extracts from strains PAT10, VD57 and FRC41 was demonstrated by the measurement of higher concentrations of cytokines IFN- $\gamma$ , IP-10, TNF, IL-2, IL-6, IL-10 and IL-17, after stimulation in the study subjects, with the exception of IL-4 in the control group. Increased production of these cytokines when stimulated with *M. tuberculosis* specific antigens was observed in the works of [12] and [13]. Rebouças et al (2011) [14], using *C. pseudotuberculosis* semi-purified antigens in samples of small ruminants with caseous lymphadenitis, showed higher levels of IFN- $\gamma$  in the group of infected animals after stimulation. PAT10 extract was able to differentiate study groups when IFN- $\gamma$  concentrations were compared. Although it is found in small amounts, IFN- $\gamma$  is currently used as a biomarker for TB [15].

Under antigenic stimuli, lower IFN- $\gamma$  levels were measured in the TB group (Table 1) compared to the control group. This may have occurred because of the short incubation time, already in the presence of mycobacterial antigens, IFN- $\gamma$  remained at undetectable concentrations before the sixth day of stimulation<sup>[16]</sup>. In addition, IFN- $\gamma$  production in response to a specific *M. tuberculosis* antigen appears to decrease in untreated TB individuals<sup>[17]</sup> which may have occurred in the present study.

Evaluating the cytokine profile in peripheral blood of patients with active TB and healthy contacts, in response to the *M. tuberculosis* 30-Kd antigen, Torres et al. (1998)<sup>[18]</sup> found lower concentrations of IFN- $\gamma$  in the TB group. The authors have suggested that IFN- $\gamma$  plays a protective role in healthy contact individuals. Deenadayalan et al (2010)<sup>[19]</sup> demonstrated the same results as in this present study.

Similarly to the IFN- $\gamma$  concentrations, IP-10 concentrations were able to differentiate the study groups, however with higher concentrations in the TB group. Thus, IP-10 may be a useful biomarker to monitor the efficacy of therapy in patients with active TB<sup>[20]</sup>, since this molecule exists in a larger amount of serum which would facilitate its laboratory dosage and clinical interpretation<sup>[21]; [22]</sup>. Several studies have shown high production of IP-10 in individuals infected with *M. tuberculosis*, allowing the development of updated and simplified test platforms<sup>[15]</sup>.

The VD57 antigen was able to differentiate study groups when compared to the concentrations of IFN- $\gamma$ . The FRC41 antigen, following the previous comparison, led to the differential production of IP-10 between the groups. The VD57 antigen stimulation allowed the differentiation of the groups when evaluating the concentrations of IFN- $\gamma$ . The culture with FRC41 antigen culminated in the differential production of IP-10 between the groups.

These extracts have potential for use in an immunodiagnostic kit, although they require complementary studies in order to identify which components of the protein extracts interact with the immune system stimulating the production of the cytokines.

#### 4.1 Acknowledgment

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## 5 | CONFLICT OF INTEREST DISCLOSURE

There are no conflicts of interest.

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## ÍNDICE REMISSIVO

### A

Agentes de Controle 84  
Alcaloides 3, 7, 12, 14, 15, 16, 18, 19, 20  
Álcool Gel 83, 84, 85, 88, 89, 90  
*Allium Cepa* 18, 19, 21, 25, 29, 30, 31, 32, 33  
antibióticos 16, 92, 99  
Antibióticos 92  
Antigenicity 58  
Antimicrobial Subinhibitory Concentrations. 67  
Antimicrobiano 1, 2, 6, 9, 12, 13, 83, 88, 89, 93  
Antissepsia 83, 84, 89  
Artemia Salina 18, 19, 21, 24, 26, 31, 33, 34

### B

*Bacteroides Fragilis* 66, 67, 68, 73, 74, 81, 82  
Bioativos 3, 18, 19, 20, 29, 31  
Bovinos 92

### C

*Corynebacterium Pseudotuberculosis* 57, 58, 59, 63, 64  
Criptococose 35, 48, 49, 50, 52, 53  
Cryptococcus Neoformans 35, 36, 40, 42, 53, 54, 55, 56  
Cytokines 58, 59, 60, 62, 63, 64

### E

Endoglucanase 101, 102, 103, 105, 110  
Exoglucanase 101, 109  
Extrato Orgânico 12

### F

Fermentação 101, 102, 104, 107  
Fitoquímica 1, 4, 7, 10, 12, 14, 15, 17

### J

Jatobá 1, 2, 3, 4, 7, 9

## M

Microbiota 81, 91, 92, 93, 95, 97

Microrganismos 7, 2, 14, 43, 44, 47, 83, 84, 85, 88, 89, 101, 102, 104, 105, 107

*Mycobacterium Tuberculosis* 57, 58, 64

## P

Pathogenicity 35, 58, 66, 67, 68, 69, 73, 76, 77, 78, 80

Plantas Aquáticas 19, 33

Plantas Medicinais 2, 3, 9, 10, 11, 17, 20, 21, 30, 31

Purificação 101, 102, 105, 106, 108

## R

Resistência 91, 92, 93, 94, 95, 96, 97, 98, 99

## S

*Staphylococcus Aureus* 1, 2, 10, 11, 14

## T

Toxicidade 12, 18, 20, 21, 22, 24, 25, 27, 29, 30, 31, 33, 34, 35, 36, 51, 52

Tracto Gastrointestinal 91, 92, 93, 95, 96

Tratamento 3, 4, 9, 13, 18, 20, 21, 25, 26, 30, 35, 36, 48, 50, 52, 67, 113

Tuberculosis 19, 57, 58, 59, 61, 62, 63, 64, 65, 113

## V

Virulência 9, 35, 36, 38, 39, 43, 44, 46, 47, 48, 113

 **Atena**  
Editora

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