www.elsevier.com/locate/jep

In vitro and In vivo antimycobacterial, hepatoprotective and immunomodulatory activity of Euclea natalensis and its mode of action

Namrita Lall, Vivek Kumar, Debra Meyer, Nomasomi Gasa, Chris Hamilton, Motlalepula Matsabisa, Carel Oosthuizen

PII: S0378-8741(16)31454-4
DOI: http://dx.doi.org/10.1016/j.jep.2016.10.060
Reference: JEP10518

To appear in: *Journal of Ethnopharmacology*

Received date: 2 August 2016
Revised date: 11 October 2016
Accepted date: 19 October 2016

Cite this article as: Namrita Lall, Vivek Kumar, Debra Meyer, Nomasomi Gasa Chris Hamilton, Motlalepula Matsabisa and Carel Oosthuizen, In vitro and I vivo antimycobacterial, hepatoprotective and immunomodulatory activity o Euclea natalensis and its mode of action, *Journal of Ethnopharmacology* http://dx.doi.org/10.1016/j.jep.2016.10.060

In vitro and *In vivo* antimycobacterial, hepatoprotective and immunomodulatory activity of *Euclea natalensis* and its mode of action

Namrita Lall [a*], Vivek Kumar [a], Debra Meyer [a,b], Nomasomi Gasa [a], Chris Hamilton [a,c], Motlalepula Matsabisa [d] and Carel Oosthuizen [a]

[a]*Department of Plant and Soil Sciences, University of Pretoria, South Africa*

[b]*Faculty of Science, University of Johannesburg, South Africa*

[c]*Department of Pharmacy, University of East Anglia, Norwich, United kingdom*

[d]*Department of Pharmacology, University of the Free State, South Africa*

*Corresponding author:

Address: Prof. Namrita Lall
Department of Plant and Soil Sciences
Plant Science Complex
University of Pretoria, Hatfield Campus
Lynwood road
Pretoria
South Africa
Tel: +27124206670

Co-authors:

Carel Oosthuizen: u04405765@tuks.co.za
Motlalepula Matsabisa: MatsabisaMG@ufs.ac.za
Vivek Kumar: vivek494@gmail.com
Debra Meyer: dmeyer@uj.ac.za
Nomasomi Gasa: nomasomi.gasa@gmail.com
Chris Hamilton: C.Hamilton@uea.ac.uk

ABSTRACT

Ethnopharmacological relevance: The Natal gwarri or Natal ebony (*Euclea natalensis* A.DC.) is a deciduous tree found widespread throughout southern Africa, especially in Kwazulu-Natal and the southern cost. It has been widely used by indigenous communities such as the Zulus, Tsongas and Vendas for symptoms related to tuberculosis (TB). The decoctions made from the plant parts are administered for chest diseases to treat complications such as chest pains, bronchitis, pleurisy and asthma. TB is prevalent in immune-compromised patients and it is evident that TB-drugs cause hepatotoxicity. The objective of the present study was therefore to evaluate the antimycobacterial activity of the

ethanolic extract of *E. natalensis* against TB and its hepatoprotective and immunomodulatory activities.

Materials and methods: The antimycobacterial, antioxidant, hepatoprotective, immunomodulatory activity and cytotoxicity of the ethanolic extract of the shoots of *E. natalensis* were determined *in vitro*. The mechanism of action of the antituberculosis activity was determined by investigating the inhibitory effect on mycothiol disulfide reductase enzyme. Furthermore, the acute, sub-acute toxicity (50-2000 mg/kg) and antimycobacterial effect (300 mg/kg) of *E. natalensis* shoot extract were investigated in Balb/c mice. Hepatoprotective activity of the extract (50-150 mg/kg) was evaluated on isoniazid and rifampicin (50 mg/kg; i.p.) induced hepatic damage in a rat model.

Results: The minimum inhibitory concentration of the extract was found to be 125 µg/ml against *Mycobacterium tuberculosis*. The extracts fifty percent inhibitory concentration (IC_{50}) against 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was found to be 22.55 µg/ml. The plant showed a hepatoprotective effect (50% at 12.5 µg/ml) and the ability to increase T-helper 1 cell cytokines; Interleukin 12, Interleukin 2 and Interferon α by up to 12 fold and the ability to decrease the T-helper 2 cell cytokine Interleukin 10 4 fold when compared to baseline cytokine production. No cellular toxicity was observed in primary peripheral blood mononuclear cells (PBMC's) and two secondary cell lines; U937 monocytes and Chang liver cells (a derivative of the HepG2 cell line). During mechanistic studies, the extract showed a 50% inhibition of mycothiol reductase activity at 38.62 µg/ml. During the acute and sub-acute studies, *E. natalensis* exhibited no toxic effect and the fifty percent lethal dose (LD_{50}) was established to be above 2000 mg/kg. The extract was able to reduce the mycobacterial load (1.5-fold reduction) in infected mice. Isoniazid and rifampicin caused significant hepatic damage in rats, and the extract was able to reduce the toxicity by 15% and 40% at 50 and 150 mg/kg respectively.

Conclusion: The present study supports the traditional usage of the plant against tuberculosis symptoms. The study showed the ability of *E. natalensis* shoot extract to inhibit mycobacterial growth, stimulate an appropriate immune response and have a hepatic protective effect. Due to the extract's significant results for hepatoprotective, immunomodulatory effects and antimycobacterial activity, it may prove to be effective to serve as an adjuvant for TB-patients.

Keywords: *Euclea natalensis*, Antimycobacterial, Toxicity, Immunomodulation, Hepatoprotection.

1. Introduction

Tuberculosis remains one of the world's deadliest infectious diseases with an annual incidence rate of more than 9 million and death rate of 1.3 million in 2014 (WHO, 2016). Termed as a disease of poverty, it is the middle and low-income countries that are affected the most.

Although effective, the current treatment regime for tuberculosis (TB) is not without complications. The time required to treat TB can range from 6-9 months. This time duration not only increases the risk of a patient infecting more individuals but also increases the risk of the patient developing drug-induced hepatic damage (Sia and Wieland, 2011). Secondly, during tuberculosis infection, an imbalance between the T-helper cell 1 (Th1) and T-helper cell 2 (Th2) human immune responses exist, where it has been shown that an increase in the Th1 response could potentially be used as an immune therapy for active TB infections (Wong et al., 2013). Thirdly, the increase in multidrug- (MDR-TB) and extensive drug (XDR-TB) resistance tuberculosis is on the rise and the development of new drugs and treatment is an on-going process (Allerberger et al., 2016; Ma et al., 2010). An adjuvant with hepatoprotective effect, antioxidant activity, which can stimulate the production of the Th1 cytokines and which can target the appropriate enzymes and mechanism to combat resistance is imperative. When developing a new drug or adjuvant, these aspects should be considered. One of the targets, in the causative organism, *Mycobacterium tuberculosis*, has been identified as Mycothiol disulfide reductase (Mtr), which is not targeted by any of the existing tuberculosis drug. Mycothiol, the product of Mtr is the low molecular weight thiol, which maintains the oxidative stress environment within the bacterium cell. This enzyme is distinct from the human analogue, glutathione reductase, making Mtr the ideal enzyme target for drug and product development (Hernick, 2013; Mdluli and Spigelman, 2006; Zhang, 2005).

Throughout history, the human civilization has been depending and relying on natural products to treat their ailments. These products were obtained from flora, fauna and mineral sources from the indigenous people's immediate surroundings and more remote areas. Nature has been the source of medicinal agents for thousands of years, and a staggering number of modern drugs have been isolated from natural sources, especially plants (Ghorbani et al., 2006). Over the last few decades, there has been a significant increase and a growing interest in alternative medicines, especially those from plant origin (Ekor, 2013; WHO, 2004). This

green revolution can be attributed to several reasons; conventional medicine can be inefficient, abusive and incorrect use of these drugs can result in adverse side effects. A large percentage of the world's population does not have access to conventional pharmacological treatment. Lastly, indigenous medicine and ecological awareness suggest that "natural" products are safe and harmless (Rates, 2001). However, this is not always the case and the use of these natural products are not always authorized by legal authorities or practitioners that are familiar with the efficacy and safety of the phytomedicine. There are many articles and published papers that report on the lack of quality and safety in the production, trade and prescription of phytomedicinal products (Ekor, 2013; Govindaraghavan and Sucher, 2015; Pelkonen et al., 2014; Raynor et al., 2011). It is clear that modern society's general views of health services, the need for new novel pharmaceutical drug development and the recognition that research in and on ethnopharmacology as an alternative, represents a viable and suitable approach for new drug development and research.

Many plants have been used traditionally to treat the symptoms of tuberculosis. According to previous research done, some plants have shown significant activity against Mycobacterium in *in vitro* studies, with a minimum inhibitory concentration (MIC) of lower than 50ug/ml. These plants include; *Berchemia discolor* with an MIC of 12.5 µg/ml, *Warbugia salutaris* (25 µg/ml), *Terminalia sericea* (25 µg/ml), *Bridelia micrantha*, and *Diospyros mespiliformis* (12.5 µg/ml) (Green et al., 2010; Mahapatra et al., 2007). *Euclea natalensis*, a plant belonging to the Ebenaceae family has been used by multiple communities in southern Africa including, Tsonga, Shangaan, Venda and the Zulu tribes. Mainly and most importantly this plant has been used for the treatment of chest complaints. Decoctions from parts of *E. natalensis* have been made to treat chest pains, bronchitis, pleurisy and asthma. It has also been used against leprosy (caused by *Mycobacterium leprosy*). Other uses include; as a dye and the shoots are chewed as a tooth cleaning instrument (Bryant, 1909). Chemical profiling of *Euclea natalensis* have produced an array of isolated compounds and the antitubercular activity has mostly been associated with the isolated naphthoquinones; 7-methyl juglone, diospyrin, isodiospyrin, neodiospyrin and shinanolone. These compounds have been isolated in higher quantities in the roots of the plant but are also present in lower quantities, in the shoots (Bapela et al., 2007). During an earlier study the MIC's of 7-methyl-juglone, diospyrin, isodiospyrin, neodiospyrin and shinanolone were found to be 0.5 µg/ml, 8.0 µg/ml, 10.0 µg/ml, 10.0 µg/ml and 100 µg/ml respectively (van der Kooy et al., 2006).

The present study investigated *E. natalensis*' ability to indirectly combat this disease by means of assessing its antimycobacterial, immunomodulatory as well as hepatoprotective activity. By combining antibacterial activity with immunomodulation a double edged sword can be produced to directly kill the bacteria and boost the human immune response to indirectly help the patient in combating the disease. The cytotoxic effect of the extract was investigated against primary peripheral mononuclear cells and secondary Chang liver and U937 cell lines *in vitro*, and acute and sub-acute toxicity in Balb/c mice, *in vivo*. The mechanism of action was evaluated on Mycothiol reductase enzyme.

2. Materials and methods

2.1. Collection, authentication, and extraction of plant

Shoots of *Euclea natalensis* A. DC. were collected from Edakeni, Kwazulu-Natal, South Africa, identified and authenticated at the H.G.W.J. Schweickerdt Herbarium (PRU 95059). The dried shoot material (5 kg) was mechanically ground to a fine powder using a 2mm sieve and extracted using 95% ethanol in a 1:10 ratio for 4 days. The extract was filtered using a Buchner funnel, and concentrated to dryness in a rotary evaporator (Büchi Rotavapor R-200) under reduced pressure and controlled temperature (37 – 40°C) and percentage yield was found to be 10%.

2.2. In vitro antimycobacterial activity

A drug-susceptible strain of *M. tuberculosis*, H37Rv (ATCC 27264) was obtained from American Type (MD) USA Culture Collection. *M. tuberculosis* was plated onto Löwenstein-Jensen medium and allowed to grow for 3-4 weeks at 37 °C. A single bacterial colony was transferred into fresh 7H9 media supplemented with 10% OADC and 2% PANTA and incubated for another 3 weeks.

A 96-well microtitre plate was used to determine the minimum inhibitory concentrations (MIC) of *E. natalensis* extract according to the method of Franzblau and collaborators (1998), with slight modifications. The extract was dissolved in 20% DMSO in sterile Middlebrook 7H9 media supplemented with 10% OADC and 2% PANTA to obtain a stock solution of 200 mg/ml. The extract was tested in triplicate in four independent assays. A two-fold serial dilution of the extract was made to yield a final concentration ranging from 1000 µg/ml to 3.13 µg/ml. Isoniazid (INH), at final concentrations ranging from 4.0 µg/ml to 0.03 µg/ml, served as the positive drug control. Control wells without the tested plant extracts and

a solvent control, DMSO at a final concentration of 5%, were included in the assays. Two hundred microliters of sterile distilled water were added to all the perimeter wells of the plate to compensate for evaporation. The plates were sealed with Parafilm and incubated at 37ºC for 5 days. Forty microliters of freshly prepared 1:1 mixture of Alamar Blue reagent and 10% Tween 80 were added to the medium control well, and re-incubated at 37ºC for 24 h. After observing a colour change from blue to pink, Alamar blue reagent was added to the rest of the test wells and incubated at 37ºC for an additional 24 h. The minimum inhibitory concentration (MIC) was defined as the lowest concentration where no colour change from blue to pink was observed.

2.3. In vitro antioxidant activity

The method of du Toit and collaborators (2001), with slight modification, was followed to determine the radical scavenging capacity (RSC) of the extract. Briefly, stock solutions of ascorbic acid and the extract were prepared at concentrations of 2 mg/ml and 10mg/ml respectively. The assay was performed in a 96-well microtitre plate. The final test concentrations ranged from 3.9 µg/ml to 500 µg/ml for the extract and 0.781 µg/ml to 100 µg/ml for ascorbic acid. Lastly, 0.04 M 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) ethanolic solution was added to each test well, except for the negative control where distilled water was used. The plate was kept in the dark for 30 minutes. The scavenging activity was determined using a BIO-TEK Power-Wave XS multi-plate reader, measuring DPPH concentrations at a wavelength of 515 nm.

2.4. In vitro cell cytotoxicity

Cytotoxicity of the extract was evaluated using the method as described by Lall and collaborators (2013). The cell lines (primary peripheral blood mononuclear cells, U937 monocytes and chang liver cells) were seeded (100 µl) in 96-well microtitre plates at a concentration of 1×10^5 cells/ml. The plates were incubated for 24 h at 37ºC and 5% CO_2 to allow the cells to adhere. The extract was dissolved in DMSO and the final test concentration of the extract ranged from 1.563 µg/ml to 400 µg/ml. The microtitre plates were incubated for a further 72 h. The control wells included vehicle treated cells exposed to 0.04% DMSO and the positive control, 'Actinomycin D' with concentrations ranging between 0.002 µg/ml to 0.5 µg/ml. After the 72 h incubation period, Prestoblue reagent (20 µl) was added and the plates were further incubated for another 2 hours. After the incubation, the absorbance of the colour complex was read at 570 nm with a reference wavelength set at 600 nm using a BIO-

TEK Power-Wave XS multi-well plate reader. The assay was performed in triplicate to calculate a fifty percent inhibitory concentration (IC_{50}) of the cell population.

2.5. In vitro immunomodulation assay

Peripheral blood mononuclear cells (PBMC's) were isolated and assessed for their cytokine production after the treatment with shoot extract. The selection criteria of volunteers/patients were based on healthy volunteers above the age of 21 with no history of any major diseases. The study was approved by the ethics committee of the Faculty of Natural and Agricultural Sciences (EC120411-046, University of Pretoria, South Africa). The PBMC's were isolated using a density gradient centrifugation utilizing Ficoll-Hypaque (Sigma, St. Louis, MO), described previously by Traoré and Meyer (2007). Briefly, the buffy coat was carefully collected and the cells were treated for 5 minutes with cell lysis buffer to remove the contamination of erithrocytes. The cells were cultured in peridishes for 1 hour at 37°C to allow the attachment of monocytes. Non adherent cells were removed by washing twice with PBS. The cells were cultured at a concentration of $1x10^5$ cells/ml in 24-well plates in RPMI 1640 medium containing 10% inactivated foetal bovine serum. To differentiate the monocytes into macrophages, 2 g/ml of PHA (Phytohaemagglutinin, Sigma-Aldrich) was added for 24 h at 37°C in a 5% CO2 incubator. Differentiated cells were treated with the extract prepared at half the IC_{50} concentrations as determined by the cytotoxicity assay. As controls, untreated cells, cells treated with solvent (0.5% DMSO) and cells treated with the positive control (Septilin at 5 µg/ml) were used. A viability test was conducted, after incubation, to determine the viability of the cells when supernatants were collected. The test plates were incubated for 18-24 hours at 37ºC with 5%CO_2. After incubation, the plates were sealed and centrifuged for 5 min at 800g. Supernatants were collected in 150 µl aliquots and used directly or stored at -72ºC.

The cell-free supernatant obtained from treated peripheral blood mononuclear cells were analysed for their immune stimulatory effects by assessing the cytokine production. Two methods were utilised; Sandwich enzyme-linked-immunosorbent-assay (ELISA) (OptEIA™ Human IL12 (p40) ELISA, BD Biosciences) for the analysis of Interleukin 12 cytokine and Cytometric bead array (Cytometric Bead Array (CBA) Human Th1/Th2, BD Biosciences) flow-cytometry for the other Th1/Th2 cytokines. The kits were used according to the manufacture's instruction.

2.6. In vitro hepatoprotective assay

Human HepG2 cells were adjusted to be 1 x 10^5 cells/ml in Eagle's minimal essential medium (EMEM) supplemented with 10% FBS, and 100 µl of the cell suspension were plated into 96-well culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator for 16 h overnight. Post incubation, the cells were treated separately with varying concentrations of extract ranging between 3.1-100 µg/ml and incubated for 24 h. Silymarin (6.25 to 25 µg/ml) was used as a positive control. After incubation, the cells were treated with 30 mM of D-galactosamine and incubated for 24 h. Thereafter, the supernatant was discarded and the cells were washed with PBS, the fresh growth medium along with 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) was added and incubated for 1 h to allow the formation of formazan crystals. Finally, the medium was removed, and the formazan crystals were dissolved using DMSO; the absorbance was measured at a wavelength of 570 nm.

2.7. In vivo acute and subacute toxicity

The animals used in the toxicity studies were approved by the Animal Use and Care Committee (AUCC T005-12). The acute and sub-acute toxicity studies were carried out on female Balb/c mice (8 weeks old) by administering the extract orally. For the acute toxicity study the mice received two single doses at different instances, according to the Organisation for Economic Co-operation and Development (OECD) guidelines for preclinical toxicity testing, at 300 and 2000 mg/kg. For the sub-acute toxicity study, a two-week study was conducted with a single dose daily at 0, 100, 200 and 300 mg/kg of the extract, according to the OECD guidelines.

2.8. In vivo antimycobacterial activity

The extract was tested for its antimycobacterial activity in *Mycobacterium tuberculosis* H37Rv infected mice. The animals used for the infection study was approved by the institutional University of Pretoria Animal Ethics Committee (EC027-13). Briefly, Balb/c mice, infected by utilizing aerosol infection at 10^6 cfu/ml, were randomly divided into four groups of five animals per group. The vehicle control group (5% DMSO), a positive control group was administered with once a day dosing of pyrazinamide and rifampicin (75 mg/kg and 1000 mg/kg per mouse, respectively). The test group received 300mg/kg per mouse of plant extract together with the positive drugs and the last group received only the plant extract at 300 mg/kg, daily. Twenty-four hours after infection 5 untreated mice were sacrificed to determine baseline infection in lungs by means of bacterial cultivation. Four weeks after TB infection another 5 untreated mice were sacrificed prior to the

commencement of treatment to determine the bacterial loads in lungs at the beginning of treatment. Dosing commenced 4 weeks after infection and continued for 4 weeks. The animals were monitored daily.

2.9. In vivo hepatoprotective effect

The hepatoprotective activity of *E. natalensis in vivo* was assessed using a rat model of Isoniazid- and rifampicin-induced hepatotoxicity according to the method of Ilyas and collaborators (2011) with slight modifications. The animals used in the hepatoprotective study were approved by the Animal Use and Care Committee (AUCC, T006-13). After 7 days of acclimation, the Sprague-Dawley rats were randomly divided into five groups consisting of six rats per group. The first group served as an untreated control group. The negative control group received only intoxicant (Isoniazid and rifampicin at 25 mg/kg intraperitoneally). Positive drug control group received Silymarin at 50 mg/kg with the intoxicant. The experimental drug groups received *E. natalensis* extract at doses of 50 and 150 mg/kg together with the intoxicant. The experimental drugs were administered orally to the groups of rats, once per day for 30 days. Twenty-four hours after the last administration, all rats in each group were anaesthetized with isoflurane and then sacrificed. Blood was collected and liver tissues were immediately removed and frozen away at -80°C until further analysis.

2.10. Mode of action of the extract

The experimental procedure as described by Hamilton and collaborators (2009) with slight modifications was used to determine the enzyme inhibition potential of the *E. natalensis* extract, on mycothiol (Mtr) reductase. Briefly, the inhibition assay with Mtr was carried out at 30ºC in a 96-well microplate, assay volume of 200 µl, with 50 mM Hepes (pH 7.6), 0.1 mM EDTA, 70 µM NADPH, 100 µM Ellman's reagent (DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid)), and substrate (60 µM). The extract was dissolved in DMSO, and the final concentrations ranged from 7.8 – 500 µg/ml. Mtr was pre-incubated with NADPH for 5 min at 30ºC before initiating the reaction by the addition of the substrate. Enzyme activity was monitored by means of the increase in absorbance at 405 nm due to the formation of TNB (5′-mercapto-2-nitrobenzoic acid) via the continuous DTNB-coupled assay. The kinetic read intervals were set to capture data every 15 seconds and the assay was left to run for 15min at a set temperature of 30°C. Initial rates were measured from the linear region of the progress curve. The IC_{50} was calculated from a 4-parameter sigmoidal regression line.

2.11. Homology modelling

Mycobacterium tuberculosis mycothiol reductase (Mtr) sequence was retrieved from UniProtKB/TrEMBL database (primary accession number A0A0T9X864) (Boeckmann et al., 2003). To identify homologous sequences with known 3D structure, a BLASTP (protein-protein Basic Local Alignment Search Tool) search was carried out against the protein data bank (PDB) (Altschul et al., 1990; Bernstein et al., 1977). It searches a database of available protein structures with sequences to identify homologous structure using a query protein sequence with no structure. A number of sequences homologous to Mtr were obtained. The top hit 1GER (*E. coli – Glutathione reductase*) showed good alignment with 30% sequence identity (abundance of exact amino acid at particular position in query and target sequences) and an E-value (expectation value: signifies error that may occur by chance) of 5e-48 with Mtr sequence. Generally, a lower E-value indicates that alignment is real. MODELLER9.16 was used for comparative protein modelling (Šali et al., 1995). Simultaneous modelling of the ligand in the Mtr protein was carried out using template 1BWC (human glutathione reductase (Gtr) complexed with ajoene inhibitor), as it can be assumed that the ligand binding modes are similar to the target and the template proteins. Accordingly, ligands were then transferred among target structures, keeping their orientation as a restraint for the subsequent modelling process. Subsequently, models were selected on the basis of high molpdf score and subjected to loop refinement and model validation using iterative evaluation of Ramachandran plot, ERRAT plot and ProSA Z-core (Colovos and Yeates, 1993; Lovell et al., 2003; Wiederstein and Sippl, 2007).

2.12. Molecular docking

Compounds previously isolated and reported from *Euclea natalensis* were selected for molecular docking on mycothiol reductase. These include; 7-methyl juglone (MIC on TB – 0.5 µg/ml), menadione (100 µg/ml), shinanolone (100 µg/ml), diospyrin (8 µg/ml) and neodiospyrin (10 µg/ml) (Bapela et al., 2007; van der Kooy et al., 2006). These compounds have also been isolated and identified in the shoots and also in the shoot extract used in this study (Bapela et al., 2007; Tannock, 1973; van der Vijver and Gerritsma, 1974). The compounds were selected based on their antimycobacterial activity (Mahapatra et al., 2007; van der Kooy et al., 2006). Molecular docking was performed using GOLD (Genetic Optimization for Ligand Docking) program (Jones et al., 1997). It uses genetic algorithm considering ligand flexibility and partial protein flexibility (Verdonk et al., 2003). The default

docking parameters were employed for docking study. It performs 100,000 genetic operations on a population size of 100 individuals and mutation rate 95. The homology model of Mtr was used for docking study. The structures of compounds were sketched using Chemdraw3D and minimized considering rmsd cut-off of 0.10Å. The docking protocol was set by extracting and re-docking the ajoene inhibitor in the Mtr homology model with rmsd ~1.0Å. It was followed by docking of compounds isolated from *E. natalensis* in the active site defined as 5Å regions around the ajoene inhibitor in Mtr protein. Further, all molecules were evaluated for possible molecular interactions with Mtr active site residues using PyMol molecular visualizer (DeLano, 2002).

2.13. Statistical analysis

All data were analysed using GraphPad Prism 4. Sigmoidal dose-response curves were analysed using a 4-parameter nonlinear regression. Tukey's multiple comparison tests were performed by one-way analysis of variance (ANOVA). A *P*-value lower than 0.05 was considered to be statistically significant. All data are expressed as Mean ± SD.

3. Results and discussion

3.1. In vitro antimycobacterial, antioxidant, cytotoxic, immunomodulation and hepatoprotective activity

The pharmacodynamics of the extract was evaluated and is summarized in Table 1. The minimum inhibitory concentration (MIC) of the *Euclea natalensis* ethanolic shoot extract on a drug-sensitive strain of *Mycobacterium tuberculosis* (H37Rv) was found to be 125 µg/ml. The positive control Isoniazid (INH) exhibited an MIC of 0.25 µg/ml. Our results were comparable to some of the studies conducted earlier. In a study conducted by Nguta et al., (2016), five selected medicinal plants were assessed for their antimycobacterial activities, one plant extract of *Solanum torvum* exhibited an MIC below 200 µg/ml, whereas the other extracts' MIC's ranged between 2500 and 10 000 µg/ml on the tested *Mycobacterium* spp. During hepatic damage caused by long exposure of TB-drugs, antioxidants play a vital role in protecting and decreasing hepatic injury. The DPPH scavenging activity of the extract was evaluated and the extract and ascorbic acid exhibited an IC_{50} of 22.55 ± 2.93 µg/ml and 4.34 ± 0.48 µg/ml, respectively. During the study conducted on the cytotoxic effect of *E. natalensis* on peripheral blood mononuclear cells, U937 monocytes and Chang liver cells, the plant extract showed low cellular toxicity on primary PBMC's (IC_{50} 131.3 ± 1.67 µg/ml),

secondary U937 monocytes (IC_{50} 208.9 ± 10.3 µg/ml) and Chang liver cells (IC_{50} 148.2 ± 8.3 µg/ml). An IC_{50} below 50 µg/ml has been considered to be moderately toxic and samples with a toxicity value higher than 100 µg/ml have been considered to be non-toxic (Vijayarathna and Sasidharan, 2012). In the study mentioned above, the cytotoxicity of the ethanolic extract of *Solanum torvum* was assessed on human fetal lung fibroblasts (MRC-5) and the IC_{50} of the extract was found to be 31.25 µg/ml (Nguta et al., 2016). The cytokine levels produced by the treated PBMC's were analysed and evaluated. The baseline production of cytokines by the isolated PBMC's was of low values, and the addition of the sample had a significant effect on the cytokine production. From Table 2 it is evident that there was an increase in all four Th1 cytokines when compared to the baseline and three out of the four Th1 cytokines when comparing to a known immune stimulant. Interleukin 2 showed the greatest increase from baseline to treatment, followed by TNFα and Interleukin 12. Interleukin 2 is involved in the activation of T lymphocytes together with Interferon, and these have been shown to limit the replication of mycobacteria (Johnson et al. 2003). Macrophage cell-proliferation, regulated by IL2, has been shown to be decreased in patients suffering from TB (Toossi et al. 1986). The ability of the extract to induce the production of these cytokines can possibly greatly aid the immune response in combating and inhibiting the proliferation of the bacteria. There was also a decrease in one of the Th2 cytokines and no effect on the other. This indicates a selectivity of the extract towards Th1 response. This suggests strongly the immunomodulatory properties of the extract.

E. natalensis extract was evaluated for its possible hepatoprotective activity against D-galactosamine. Drug-induced liver injury (DILI) remains a problem related to TB drug therapy, especially in TB/HIV co-infection, with damage occurring as early as 3 months after the start of the treatment (Jong et al., 2013). A significant decrease in cell viability was observed upon treatment of HepG2 cells with D-galactosamine (30 mM). *Euclea natalensis* exhibited a significant protection (50±5.9% protection) at a concentration of 12.5 µg/ml towards cell toxicity of D-Galactosamine. The reference standard, silymarin demonstrated a significant increase in cell viability against D-galactosamine-induced toxicity with a protection of 58±6% obtained at 12.5 µg/ml. The mechanism of action on mycothiol reductase showed a high inhibition with an IC_{50} of 38.62 µg/ml (Fig 1)

Table 1
Effect of *Euclea natalensis* extract on *Mycobacterium tuberculos*is inhibition, DPPH scavenging, cytotoxicity, and hepatic protection against drug-induced toxicity in human HepG2 hepatocytes.

Sample & reference standard	Antibacterial	Antioxidant	Cytotoxicity			Hepatoprotective
			IC_{50}[b] (µg/ml) ± SD			
	MIC[a] (µg/ml)	IC_{50}[b] (µg/ml) ± SD	PBMC[c]	U937[d]	Chang Liver	% Protection (12.5µg/ml) ± SD
E. natalensis shoot extract	125	22.6 ± 2.9	131.3 ± 1.7	208.9 ± 10.3	148.2 ± 8.3	50 ± 5.9
Isoniazide[e]	0.25	-	-	-	-	-
Ascorbic acid[f]	-	4.34 ± 0.5	-	-	-	-
Actinomycin D[g]	-	-	$1.3x10^{-3}$	$1.3x10^{-2}$	$8.2x10^{-3}$	-
Sylimarin[h]	-	-	-	-	-	58 ± 6.0

[a] Minimum inhibitory activity, [b] half maximal inhibitory concentration, [c] Peripheral blood mononuclear cells, [d] human macrophages, [e] antimycobacterial standard, [f] Antioxidant standard, [g] Cytotoxicity standard, [h] Hepatoprotective standard, data are represented as Mean ± SD, n=3, ANOVA p-value < 0.05

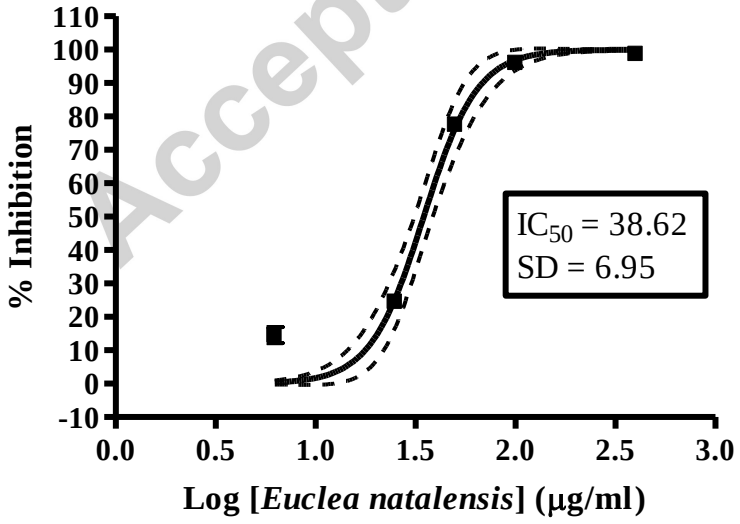

Fig. 1. Effect of *Euclea natalensis* on the inhibition of mycothiol reductase enzyme. Data are expressed as Log Mean, n=3, 4-parameter nonlinear regression.

Table 2
Effect of *Euclea natalensis* on cytokine production and excretion by treated isolated peripheral blood mononuclear cells (Th1: IL12, IL2, TNF and IFN. Th2: IL4 and IL10).

	Sample	Cell baseline production	*E.natalensis* 5 µg/ml	Septilin 5 µg/ml	Fold increase or decrease from baseline
Th1[a] (pg/ml)	Interleukin 12	17.27	75.68	59.35	4.38 x increase
	Interleukin 2	0.0	25.88	7.39	>200 x increase
	TNFα [c]	5.13	66.5	96.95	12.96 x increase
	Interferon	11.76	27.18	9.1	2.31 x increase
Th2[b]	Interleukin 4	0.0	0.0	0.0	-
	Interleukin 10	8.96	2.34	7.18	3.8 x decrease

[a]T-helper 1 cytokines, [b]T-helper 2 cytokines, [c]Tumor necrosis factor α

3.2. Mycothiol reductase structure prediction using Homology modelling

Mtr plays an important role in oxidative stress management and is oxidised to the symmetrical disulfide in the process. Mtr monomer has an amino acid sequence length of 459. To identify homologous sequences of Mtr that could be used as a template for model building, a BLASTP search against PDB using Mtr as query sequence was performed. The top hit from BLASTP, viz. *E. coli* - *Glutathione reductase* (PDB code: 1GER) has a crystal structure resolution of 1.86Å, and exhibits 30% sequence identity (Fig. 2). Another, human Gtr structure (PDB code – 1BWC) was taken for ligand modelling in the Mtr homology model. It has a crystal structure resolution of 2.1Å and showed 29% identity with Mtr sequence. This strategy was applied to identify the active site residues around the 6Å region of bound inhibitor in 1BWC. Subsequently, mapping these residues in the multiple sequence alignment led to hot spot identification in Mtr protein. The final model was validated using various tools:

(1) Ramachandran plot emphasized that Φ (phi) and Ψ (psi) dihedral angle distribution of all the amino acids residues in various regions i.e. 89.9% in the core region, 8.3% in allowed region and 1.8% in the disallowed region, as presented in Table 3 and Fig S 1respectively. This indicated that the backbone dihedral angles in the Mtr model were reasonably accurate.

(2) An ERRAT plot showed non-bonded interactions between different atom types of amino acid. It is helpful to determine the 'structure error' at each residue in the 3D structure of the protein. It provides an indication for loop refinement at those regions where the plot bar exceeds the 95% error value in ERRAT plot, shown in Fig S 2. Loop refinement was performed in several cycles, taking a few amino acids in each step and validating through

ERRAT plot. ERRAT plot of the loop refined model showed an increase in the overall score for structural quality to 96% (Table 3).

(3) ProSA-web reveals that the ProSA Z-score value is −5.83. It indicated that predicted model is in the range of native conformations of the crystal structures, as shown in Table 3 and Fig S 3.

(4) RMSD validation criteria include the superimposition of the overall structure and active site regions surrounding ajoene inhibitor in both Mtr model and Human Gtr (PDB: 1BWC). An RMSD of Cα-backbone for the overall structure was calculated to be 2.5Å (Table 1). Further, superimposition of the active site residues within the 5.0Å region surrounding ajoene inhibitor (ball and stick) in Mtr (green) and Human Gtr (cyan) showed that ~50% of the active site residues were conserved, except that Gly12, Asn15, Leu18, Leu40, Gly95 and Asn317 of Mtr were replaced with Ser30, Ala34, Arg37, Val59, Tyr114, and Ile343 in Human Gtr respectively (Fig. 2). See appendix A for the supplementary Figures (Fig S1-S3)

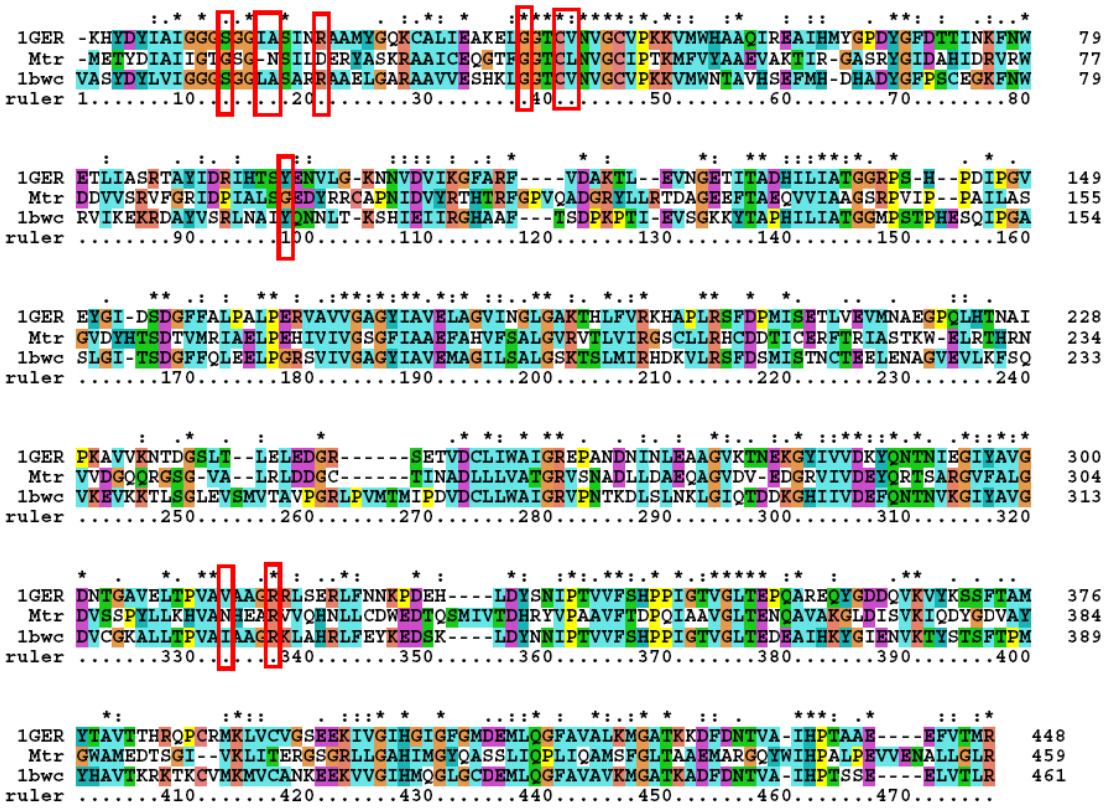

Fig. 2. Multiple sequence alignment for building Mtr homology model.

Table 3
Validation statistics for developed Mtr homology model

Model validation statistics	1GER (Template)	Mtr (Model)
% Amino acids in favoured regions	97.10	89.9
% Amino acids in allowed regions	2.9	8.3
% Amino acids in disallowed regions	0	1.8
Errat overall quality factor	97.47	96.00
ProSA Z-score	-10.99	-5.83
RMSD with template		2.5Å

3.3. Molecular docking analysis

Molecular docking was carried out to predict the binding mode of the extract constituting compounds in the Mtr active site. A fitness score is a Gold score which is used to rank the various docked poses of small molecules. It is derived using the geometrical properties and bonding affinities of ligand. All compounds were docked in the active site of Mtr successfully. The ajoene inhibitor is present as a co-crystal ligand in 1BWC structure. Despite none H-bond interactions at the Mtr active site residues, it showed highest fitness score of 44.69 as compared to rest of the compounds. Probably, it may be due to the good complementary fitting of this compound into the Mtr active site which makes balanced van der Waals interactions with surrounding residues and FAD (flavin adenine dinucleotide) (Fig. 3).

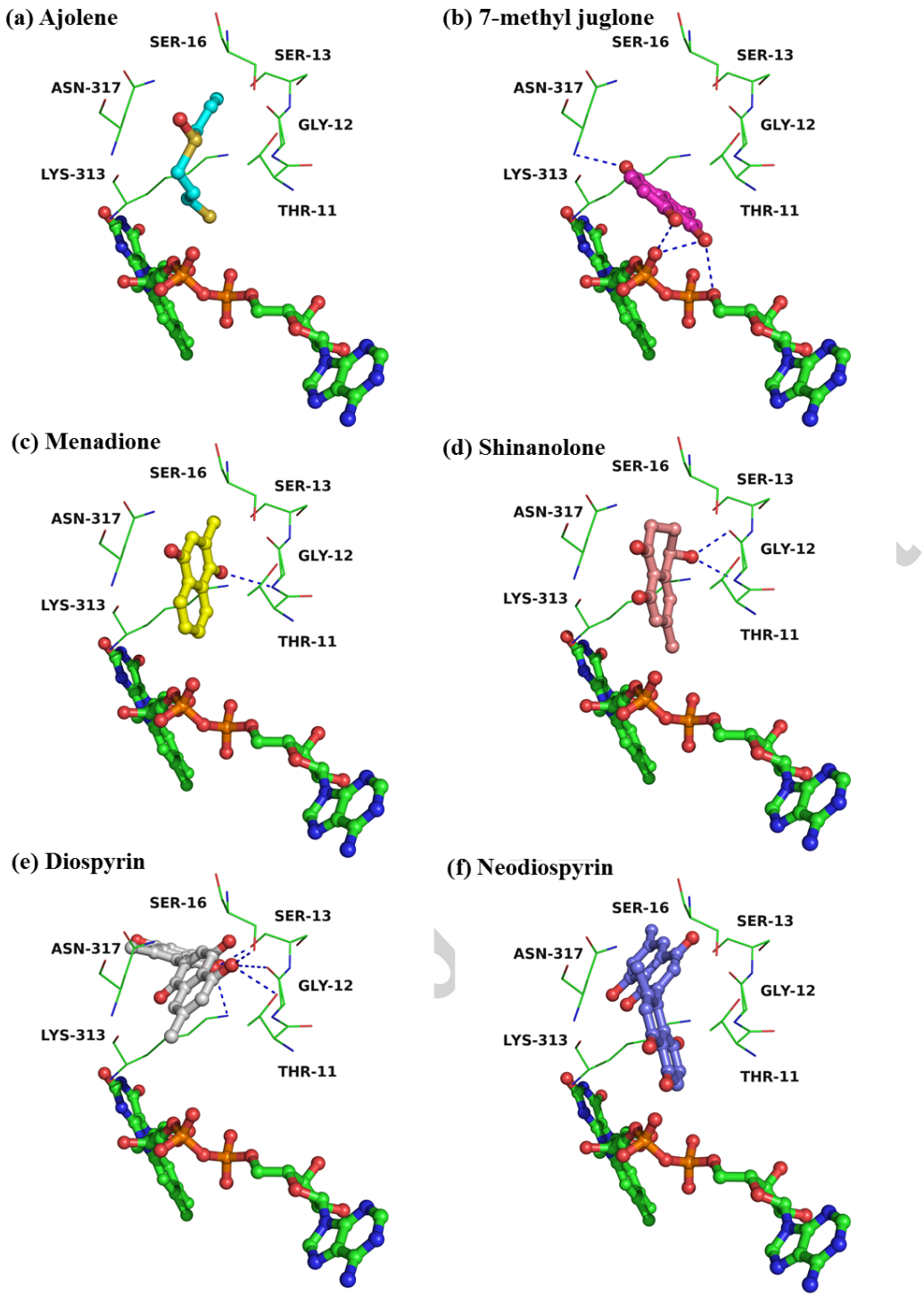

Fig. 3. Interaction of the docked compounds in the Mtr active site. FAD is shown as a green stick and ball model.

It is followed by the 7-methyl juglone which showed a fitting score of 41.10. It showed three H-bond interactions with FAD and one H-bond interaction with residue Asn317. It makes very close interaction with FAD as shown in Fig 2. Similar to 7-methyl juglone, menadione and shinanolone also formed close interactions with FAD and showed fitting

scores of 40.26 and 34.22 respectively. Also, the menadione showed one H-bond interaction with backbone atom of Gly12 while shinanolone showed two H-bond interactions with Gly12. The diospyrin and neodiospyrin showed lowest fitting scores of 26.20 and 24.51 in the Mtr active site cavity respectively. This may be due to the bulkiness of these compounds which make them dock little away from the region where other make generous fit. It is also evident from the Fig.2 where these compounds showed different binding orientation in the Mtr active site. Thus, molecular docking study indicated the importance of small molecular structures which are supposed to make proper interactions into the Mtr active site cavity.

3.4. In vivo acute and sub-acute toxicity

The initial acute toxicity study indicated that a single dose (2000 mg/kg), administered orally, did not produce any mortality, toxicity signs or behavioural changes when compared to the control group. The dosages were initiated with a single dose of 300 mg/kg and increased to 2000 mg/kg when no toxicity was observed. As per acute toxicity results, doses of 250, 150 and 50 mg/kg were used in the 2-week long sub-acute study. No unscheduled death, clinical or histopathological signs of toxicity were observed. Body weight and organ weight showed no sign of toxicity related to dose response. It was established that the LD_{50} is above 2000 mg/kg.

3.5. In vivo antimycobacterial

The antimycobacterial activity of *E. natalensis* shoot extract was evaluated on *Mycobacterium Tuberculosis* (H37Rv) infected Balb/c mice (8-week old). There was a substantial decrease in bacterial loads when comparing the infected control group to the treatment groups, showing a decrease in lung homogenate colony forming units from $1.5x10^6$ (Control) to $7.1x10^3$ (Drug control) (Fig. 4.) The extract showed a significant decrease in mycobacterial loads when used on its own and in combination with Pyr/RIF (Pyrazinamide, 75 mg/kg; Rifampicin 1000 mg/kg), reducing the load to $4.8x10^3$ cfu/ml in the lung homogenate. No toxicity was observed which could be associated with *E. natalensis* administered together with isoniazid and rifampicin.

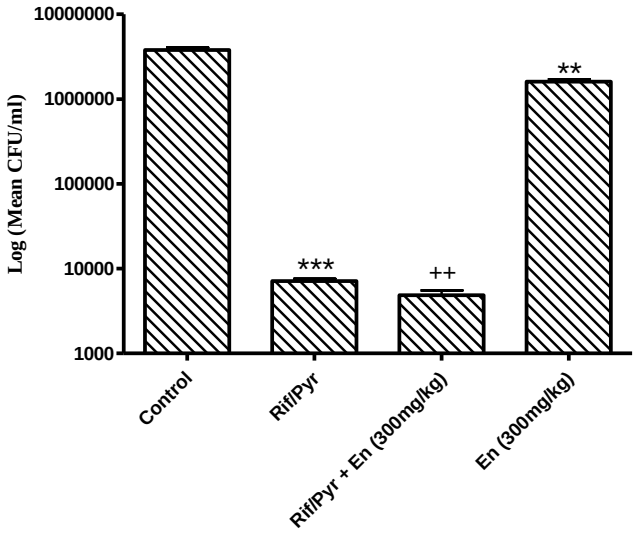

Fig. 4. Antimycobacterial effect of *E. natalensis* extract on the reduction of *Mycobacterium tuberculosis* load in infected Balb/c mice Rifampicin (75 mg/kg; p.o.) and Pyrazinamide (1000 mg/kg; p.o.) was used as the drug control. Data are expressed as Log Mean±SD, n=5; ** $p< 0.01$, when compared to the control group; *** $p< 0.001$, when compared to the control and En group; ++ $p< 0.001$ when compared to the control, $p< 0.005$ when compared with the drug control.

3.6. In vivo hepatoprotective activity

One liver enzyme Alanine transaminase (ALT) showed significant changes regarding its concentration among different groups of animals when ANOVA statistics was used. Serum levels of ALT were analysed after 14 days of treatment Isoniazid and rifampicin (comb. 50mg/kg; i.p.) significantly increased the serum levels, indicating an induced toxicity as shown in Fig. 5. Silymarin (positive control; 50 mg/ml; p.o.) was able to reduce the levels by almost half. *E. natalensis* shoot extract showed a positive dose-response in decreasing the levels of serum ALT. At a dosage of 50 mg/kg, it was capable of decreasing the levels by 15% and at 100mg/ml by almost 40%. There exists a link between antioxidant activity and hepatoprotective activity. Due to the effective ability of the extract to scavenge free radicals, this can contribute to the protection effect and the ultimate reduction of ALT and other toxic liver markers.

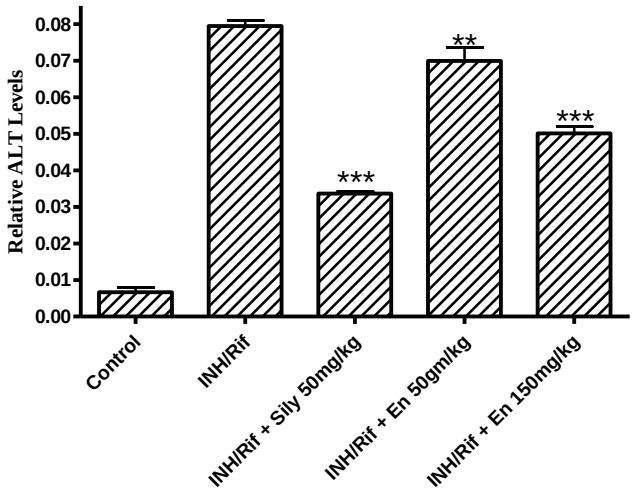

Fig. 5. The hepatoprotective effect of *E. natalensis* extract on the reduction of ALT levels in INH/Rif toxicity induced Sprague-Dawley rats. Isoniazid (25 mg/kg; i.p.) and Rifampicin (25 mg/kg; i.p.) were used as the toxic inducer, Silymarin (50 mg/kg; p.o.) was administered as the positive control. Data are expressed as Mean±SD, n=6; ANOVA; ** $p< 0.05$, when compared to the toxic (INH/Rif) group; *** $p< 0.001$, when compared to the toxic (INH/Rif) and En 100 mg/kg group.

4. Conclusion

It is conceivable that *Euclea natalensis* plant extract has shown remarkable biological activity, not only antibacterial activity but also an immune stimulatory effect as well as the protective effect on liver against toxic inducers. The molecular docking study showed that the biological activity is contributed by various compounds present in the extract. However, on the basis of fitting score, 7-methyl juglone is predicted to contribute more towards biological activity as compared to menadione and shinanolone. *E. natalensis* shows to be a promising adjuvant for patients suffering from Tuberculosis or related symptoms, and the extract should be further tested in clinical studies.

Acknowledgements

The authors are thankful to the Medical Research Council (PTA) of South Africa for the use of their Bio-safety level II and III laboratories. The bio-medical research centre, Onderstepoort, UP for their guidance and support throughout the *in vivo* studies. This work was supported by the Department of Science and Technology, African Traditional Medicine flagship project [DST/CON0164/2010].

References

Allerberger, F., Cambau, E., Lange, C., 2016. Joint efforts urgently needed at times of emerging tuberculosis drug resistance. Clin. Microbiol. Infect.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410. doi:10.1016/S0022-2836(05)80360-2

Bapela, M.J., Lall, N., Isaza-Martinez, J.H., Regnier, T., Marion Meyer, J.J., 2007. Variation in the content of naphthoquinones in seeds and seedlings of Euclea natalensis. South African J. Bot. 73, 606–610. doi:10.1016/j.sajb.2007.06.003

Bernstein, F.C., Koetzle, T.F., Williams, G.J.B., Meyer, E.F., Brice, M.D., Rodgers, J.R., Kennard, O., Shimanouchi, T., Tasumi, M., 1977. The Protein Data Bank. A Computer-Based Archival File for Macromolecular Structures. Eur. J. Biochem. 80, 319–324. doi:10.1111/j.1432-1033.1977.tb11885.x

Boeckmann, B., Bairoch, A., Apweiler, R., Blatter, M.-C., Estreicher, A., Gasteiger, E., Martin, M.J., Michoud, K., O'Donovan, C., Phan, I., Pilbout, S., Schneider, M., 2003. The SWISS-PROT protein knowledgebase and its supplement TrEMBL in 2003. Nucleic Acids Res. 31, 365–370. doi:10.1093/nar/gkg095

Bryant, A.T., 1909. Zulu Medicine and medicine-men. South. African Humanit. 2, 1–103.

Colovos, C., Yeates, T.O., 1993. Verification of protein structures: Patterns of nonbonded atomic interactions. Protein Sci. 2, 1511–1519. doi:10.1002/pro.5560020916

DeLano, L.W., 2002. The PyMOL molecular graphics system [WWW Document].

du Toit, R., Volsteedt, Y., Apostolides, Z., 2001. Comparison of the antioxidant content of fruits, vegetables and teas measured as vitamin C equivalents. Toxicology 166, 63–69. doi:10.1016/S0300-483X(01)00446-2

Ekor, M., 2013. The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. Front. Pharmacol. 4, 177. doi:10.3389/fphar.2013.00177

Franzblau, S.G., Witzig, R.S., McLaughlin, J.C., Torres, P., Madico, G., Hernandez, A., Degnan, M.T., Cook, M.B., Quenzer, V.K., Ferguson, R.M., Gilman, R.H., 1998. Rapid, low-technology MIC determination with clinical Mycobacterium tuberculosis isolates by using the microplate Alamar Blue assay. J. Clin. Microbiol. 36, 362–6.

Ghorbani, A., Naghibi, F., Mosaddegh, M., 2006. Ethnobotany, Ethnopharmacology and Drug Discovery. Iran. J. Pharm. Sci. Spring 2, 109–118.

Govindaraghavan, S., Sucher, N.J., 2015. Quality assessment of medicinal herbs and their extracts: Criteria and prerequisites for consistent safety and efficacy of herbal medicines. Epilepsy Behav. 52, 363–371.

Green, E., Samie, A., Obi, C.L., Bessong, P.O., Ndip, R.N., 2010. Inhibitory properties of selected South African medicinal plants against Mycobacterium tuberculosis. J. Ethnopharmacol. 130, 151–157. doi:10.1016/j.jep.2010.04.033

Hamilton, C.J., Finlay, R.M.J., Stewart, M.J.G., Bonner, A., 2009. Mycothiol disulfide

reductase: a continuous assay for slow time-dependent inhibitors. Anal. Biochem. 388, 91–96.

Hernick, M., 2013. Mycothiol: a target for potentiation of rifampin and other antibiotics against Mycobacterium tuberculosis. Expert Rev. Anti. Infect. Ther. 11, 49–67. doi:10.1586/eri.12.152

Ilyas, N., Sadiq, M., Jehangir, A., 2011. Hepatoprotective effect of garlic (Allium sativum) and milk thistle (silymarin) in isoniazid induced hepatotoxicity in rats. Biomedica 27, 166–170.

Jones, G., Willett, P., Glen, R.C., Leach, A.R., Taylor, R., 1997. Development and validation of a genetic algorithm for flexible docking. J. Mol. Biol. 267, 727–748. doi:10.1006/jmbi.1996.0897

Lall, N., Henley-Smith, C.J., De Canha, M.N., Oosthuizen, C.B., Berrington, D., 2013. Viability Reagent, PrestoBlue, in Comparison with Other Available Reagents, Utilized in Cytotoxicity and Antimicrobial Assays. Int. J. Microbiol. 2013, 420601. doi:10.1155/2013/420601

Lovell, S.C., Davis, I.W., Arendall, W.B., de Bakker, P.I.W., Word, J.M., Prisant, M.G., Richardson, J.S., Richardson, D.C., 2003. Structure validation by Cα geometry: ϕ,ψ and Cβ deviation. Proteins Struct. Funct. Bioinforma. 50, 437–450. doi:10.1002/prot.10286

Ma, Z., Lienhardt, C., McIlleron, H., Nunn, A.J., Wang, X., 2010. Global tuberculosis drug development pipeline: the need and the reality. Lancet 375, 2100–2109. doi:10.1016/S0140-6736(10)60359-9

Mahapatra, A., Mativandlela, S.P.N., Binneman, B., Fourie, P.B., Hamilton, C.J., Meyer, J.J.M., van der Kooy, F., Houghton, P., Lall, N., 2007. Activity of 7-methyljuglone derivatives against Mycobacterium tuberculosis and as subversive substrates for mycothiol disulfide reductase. Bioorg. Med. Chem. 15, 7638–7646. doi:10.1016/j.bmc.2007.08.064

Mdluli, K., Spigelman, M., 2006. Novel targets for tuberculosis drug discovery. Curr. Opin. Pharmacol. 6, 459–467. doi:10.1016/j.coph.2006.06.004

Nguta, J.M., Appiah-Opong, R., Nyarko, A.K., Yeboah-Manu, D., Addo, P.G.A., Otchere, I., Kissi-Twum, A., 2016. Antimycobacterial and cytotoxic activity of selected medicinal plant extracts. J. Ethnopharmacol. 182, 10–15. doi:10.1016/j.jep.2016.02.010

Pelkonen, O., Xu, Q., Fan, T.-P., others, 2014. Why is research on herbal medicinal products important and how can we improve its quality. J Tradit Complement Med 4, 1–7.

Rates, S.M.., 2001. Plants as source of drugs. Toxicon 39, 603–613. doi:10.1016/S0041-0101(00)00154-9

Raynor, D.K., Dickinson, R., Knapp, P., Long, A.F., Nicolson, D.J., 2011. Buyer beware? Does the information provided with herbal products available over the counter enable safe use? BMC Med. 9, 94. doi:10.1186/1741-7015-9-94

Šali, A., Potterton, L., Yuan, F., van Vlijmen, H., Karplus, M., 1995. Evaluation of comparative protein modeling by MODELLER. Proteins Struct. Funct. Genet. 23, 318–326. doi:10.1002/prot.340230306

Sia, I.G., Wieland, M.L., 2011. Current concepts in the management of tuberculosis. Mayo Clin. Proc. 86, 348–61. doi:10.4065/mcp.2010.0820

Tannock, J., 1973. Naphthaquinones from Diospyros and Euclea species. Phytochemistry 12, 2066–2067. doi:10.1016/S0031-9422(00)91546-2

Traoré, H.N., Meyer, D., 2007. Necrosis of host cells and survival of pathogens following iron overload in an in vitro model of co-infection with human immunodeficiency virus (HIV) and Mycobacterium tuberculosis. Int. J. Antimicrob. Agents 29, 465–470.

van der Kooy, F., Meyer, J.J.M., Lall, N., 2006. Antimycobacterial activity and possible mode of action of newly isolated neodiospyrin and other naphthoquinones from Euclea natalensis. South African J. Bot. 72, 349–352. doi:10.1016/j.sajb.2005.09.009

van der Vijver, L.M., Gerritsma, K.W., 1974. Naphthoquinones of Euclea and Diospyros species. Phytochemistry 13, 2322–2323. doi:10.1016/0031-9422(74)85052-1

Verdonk, M.L., Cole, J.C., Hartshorn, M.J., Murray, C.W., Taylor, R.D., 2003. Improved protein-ligand docking using GOLD. Proteins Struct. Funct. Bioinforma. 52, 609–623. doi:10.1002/prot.10465

Vijayarathna, S., Sasidharan, S., 2012. Cytotoxicity of methanol extracts of Elaeis guineensis on MCF-7 and Vero cell lines. Asian Pac. J. Trop. Biomed. 2, 826–829. doi:10.1016/S2221-1691(12)60237-8

WHO, 2016. Fact sheet N°104.

WHO, 2004. WHO guidelines on safety monitoring of herbal medicines in pharmacovigilance systems.

Wiederstein, M., Sippl, M.J., 2007. ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. Nucleic Acids Res. 35, W407–10. doi:10.1093/nar/gkm290

Wong, E.B., Cohen, K.A., Bishai, W.R., 2013. Rising to the challenge: new therapies for tuberculosis. Trends Microbiol. 21, 493–501. doi:10.1016/j.tim.2013.05.002

Zhang, Y., 2005. THE MAGIC BULLETS AND TUBERCULOSIS DRUG TARGETS. Annu. Rev. Pharmacol. Toxicol 45, 529–64. doi:10.1146/annurev.pharmtox.45.120403.100120

Graphical abstract