Parasite Epidemiology and Control 12 (2021) e00201

Contents lists available at ScienceDirect

Parasite Epidemiology and Control

journal homepage: www.elsevier.com/locate/parepi

The potential role and apoptotic profile of three medicinal plant extracts on *Leishmania tropica* by MTT assay, macrophage model and flow cytometry analysis

Mozhde Ilaghi [a], Iraj Sharifi [a,*], Fariba Sharififar [b], Fatemeh Sharifi [c], Razieh Tavakoli Oliaee [a], Zahra Babaei [a], Manzume Shamsi Meimamandi [d], Alireza Keyhani [a], Mehdi Bamorovat [a]

[a] Leishmaniasis Research Center, Kerman University of Medical Sciences, Kerman, Iran
[b] Herbal and Traditional Medicines Research Center, Department of Pharmacognosy, Kerman University of Medical Sciences, Kerman, Iran
[c] Research Center of Tropical and Infectious Diseases, Kerman University of Medical Sciences, Kerman, Iran
[d] Physiology and Pharmacology Department, Kerman Medical School, Kerman University of Medical Sciences, Kerman, Iran

ARTICLE INFO

Article history:
Received 19 September 2020
Received in revised form 17 November 2020
Accepted 2 January 2021

Keywords:
Medicinal plants
Leishmania tropica
Leishmanicidal activity
MTT assay
Apoptosis role

ABSTRACT

Introduction: Treatment of leishmaniasis with conventional synthetic drugs is a major global challenge. This study was designed to explore the leishmanicidal activity and apoptotic profile of three leaf extracts on *Leishmania tropica* stages.

Methods: The plants of *Quercus velutina, Calotropis procera* and *Nicotiana tabacum* were gathered from Anbarabbad county, in the southeastern part of Kerman province and extracted by maceration method using methanol alcohol. Various concentrations of the extracts (1, 10, 100 and 1000 μg/mL) were used against L. *tropica* stages to evaluate the inhibitory effect by colorimetric assay, macrophage model and flow cytometry. The MTT assay was conducted to determine the IC_{50} and CC_{50} values in promastigotes and J774-A1 macrophages, respectively. For intra-macrophage amastigotes, the leishmanicidal activity was evaluated by calculating the mean number of amastigotes in each macrophage and also IC_{50} values. The promastigote or amastigote stages with no drug and complete medium without organisms were considered as positive and negative controls, respectively. Meglumine antimoniate (Glucantime) was also used as standard drug. Also, annexin V was used to assess the apoptotic profile. All treatment settings were incubated for a standard time of 72 h in triplicates. Data were analyzed by *t*-test and ANOVA.

Results: The findings showed that all plant extracts inhibited the proliferation rate of promastigotes and amastigotes ($P < 0.001$); especially, *Q. velutina* represented the lowest IC_{50} in both stages. Besides, *Q. velutina* showed the least number of amastigotes in each macrophage compared to the other groups (4.5 μg/mL). The percentage of parasitic apoptosis at 1000 μg/mL of *Q. velutina, C. procera, N. tabacum* and Glucantime® were 37.4, 18.6, 8.5 and 52.4, respectively. Amastigotes (clinical stage) were significantly more susceptible to extracts and also Glucantime® than promastigotes ($P < 0.001$).

Conclusions: This study revealed that all three extracts of *Q. velutina, C. procera and N. tabacum* exhibited an effective antileishmanial activity and induced apoptosis against the L. *tropica* promastigotes. Further investigations are essential to isolate and analyze the chemical compositions and their biological properties.

* Corresponding author at: 22 Bahman Boulevard, Pajouhesh Square, School of Medicine, Department of Medical Parasitology and Mycology, Leishmaniasis Research Center, Kerman University of Medical Sciences, Kerman, Iran.
E-mail address: iraj.sharifi@yahoo.com. (I. Sharifi).

https://doi.org/10.1016/j.parepi.2021.e00201

1. Introduction

Leishmaniasis, a complex protozoan disease which is transmissible between animals and human (*WHO technical report series.*, 2010). According to the WHO around 12 million people are currently infected with leishmaniasis in 98 countries, over one billion people are at risk and the annual incidence is 0.7–1.2 million (Alvar et al., 2012). Clinical presentations of the disease are diverse, although two classical forms including visceral leishmaniasis (VL or kala-azar) and cutaneous form (CL) are predominant (*WHO technical report series.*, 2010). Cutaneous leishmaniasis, as the most wide-spread and neglected tropical disease consists of approximately 75% of the total case counts. The 6 countries that indicated the highest prevalence of CL including Afghanistan, Algeria, Brazil, Colombia, Iran and Syria (Alvar et al., 2012).

At present, synthetic agents such as pentavalent antimonies (Glucantime® and Pentostam®) and various other drugs such as amphotericin B, paromomycin, miltefosine, pentamidine, allopurinol and azole deravatives are used as the first-line and/or second-line alone or in combination for treatment of different types of leishmaniasis. However, the application of these drugs is limited due to low efficacy, high toxicity index, and induction of parasite resistance, long duration of action and high costs (Croft et al., 2006). Such limitations for use of chemical drugs emphasize an urgent need for discovery of new medicinal alternatives with different mechanism of action, greater potency, shorter treatment cycles, inexpensiveness and availability in endemic countries (Lamidi et al., 2005).

There are varieties of biologically active compounds with variable therapeutic potentials in herbal medicines (Mahmoudvand et al., 2015; Moein et al., 2017). The use and investigations of medicinal drug remedies on parasites, particularly against *Leishmania* species have long been well documented by various authors globally (Barati et al., 2014; Bajaj et al., 2018). In the present study, among over 15 plants previously screened, three medicinal plant extracts with high index of activity against L. *tropica* stages including *Quercus velutina, Calotropis procera* and *Nicotiana tobacum* were used.

C. procera is a species of flowering plant in the family *Apocynaceae* which is used for treatment of intestinal worm, could serve as a source of antidiabetic agent and skin diseases. *Q. velutina*is a medicinal plant in the family of *Fagaceae* that is often used as wound disinfectant, treatment of genital infections and acne. *N. tobacco* is an herbaceous plant in the family of *Solanaceae* which is cultivated worldwide. According to the WHO nearly one billion people; an estimate of 14% smoke tobacco globally. The main compositions are primarily alkaloids including nicotine, nicotiana, nicotinine and nicoteine. This herb, despite various adverse effects which is banned in many countries, is traditionally used as a relaxant, in chronic asthma, sedative, diuretic, pain reliever, expectorant, wormicidal, antiseptic and sialagogue (Schulz et al., 2001).

Apoptosis, as programmed cell death (PCD) is evolved for regulation of cell growth and development in kinetoplastids including *Leishmania* species similar to other eukaryotes (Bruchhaus et al., 2007; Rodrigues et al., 2017). This process is an essential effector mechanism which can play a crucial role to trigger innate and adaptive cell responses against the parasite forms. Further elucidation of the PCD in *Leishmania* organisms could provide valuable information on biochemical pathways and pathological mechanisms. This could be used to target new drugs for growth limitation of the causative agents and in turn control of the diseases. A typical procedure for detecting phosphatidylserine (PS) is the use of the annexin V. It is a calcium-dependent phospholipid-binding protein which attaches with high affinity to PS of apoptotic promastigotes as demonstrated by flow cytometry (Doroodgar et al., 2016).

The aim of this study was to investigate the effect of antileishmanial activities of three medicinal plant extracts on promastigotes and amastigotes (clinical stage) of L. *tropica* by colorimetric assay and macrophage model, respectively. This study was also designed to analyze the apoptotic profile of the parasite with PI Annexin V by flow cytometry analysis.

2. Material and methods

2.1. Plant extracts

In the present study, all three plants were gathered from the southeastern part of Kerman province (Anbarabbad county). The identity of the plants was approved by Professor Fariba Sharififar, Dept. of Pharmacognosy, Faculty of Pharmacy, Kerman University of Medical Sciences. All extracts were prepared by maceration method (Azwanida, 2015). For each plant, dried leaves were soaked in the methanol, as the solvent, and the system was allowed to stand for three days along with occasional shaking. The materials were thoroughly strained and the maceration processes were performed at room temperate (25 °C). The liquid extracts were allowed to dry in form of powder for use in experimental settings.

2.2. Control drug

Glucantime® (meglumine antimoniate, MA), as a drug of choice (standard control) for treatment of all clinical forms of leishmaniasis, was prepared from the Center for Diseases Control, Ministry of Health, Iran (manufactured by Sanofi-Aventis, France).

2.3. Parasite and cell culture

In this research, the Iranian strain of L. *tropica* promastigotes (MHOM/IR/75/Mash2) were cultured in 25 ml flask with RPMI-1640 (Roswell Park Memorial Institute, Biosera) medium, 10% FBS (fetal bovine serum, Biosera) and 2% penicillin and streptomycin at 24 °C incubator. Murine macrophage cells (J774-A1, ATCC no., TIB-67) were purchased from Pasteur Institute of Iran (Tehran, Iran) and cultured in complete medium of RPMI-1640, 10% FBS and 5% penicillin and streptomycin at 37 °C in 5% CO_2 atmosphere.

2.4. Cytotoxicity assay

For evaluation of cytotoxicity on murine macrophages, we determined the CC_{50} (cytotoxicity concentration for 50% of cells) of extracts on macrophages. Murine cells were grown at 10^6 cells /mL in 96- well micro plates (Lab-Tek, Nunc, USA) and allowed to adhere for 24 h at 37 °C and 5% CO_2. The non-adherent cells were removed by washing with medium, and 190 μl RPMI-1640 complete medium was added to each well of cells and incubated at the same conditions as mentioned. After that, macrophages were exposed with 10 μl of different concentrations of extracts (1–1000 μg/mL) and incubated at 37 °C for 72 h. Finally, the cytotoxicity level was evaluated using the colorimetric assay with MTT test as earlier defined in the promastigote susceptibility assay.

2.5. Anti-promastigote inhibitory effect

To evaluate the effects of these plant extracts on L. *tropica* promastigotes, colorimetric cell viability assay was used for estimation of viable cells in multi-well micro plates as described elsewhere (Shokri et al., 2012). The MTT solution (3-(4, 5-Dimethylthiazol-2-yl)-2, Diphenyltetrazolium Bromide, Sigma Aldrich, USA) consists of tetrazolium which is positively charged and is able to readily penetrates viable cells to reduce the soluble form of MTT into a colored insoluble formozan. Briefly, 100 μl of logarithmic phase promastigotes (5×10^5 cells/ml) were added to a 96-well tissue culture plate. Then, 100 μl of various concentrations of three methanolic extracts (dissolved in RPMI-1640 medium) and MA reference drug (1, 10, 100 and 1000 μg/mL) was added to each well and incubated at 25 ± 1 °C for 48 h. Afterward, 10 μl of MTT solution (5 mg/mL) was added to each well following incubation at 25 °C for 4 h. For producing a purple color and stop the reaction, cold isopropanol was added to formazan crystals, as a solvent. Finally, the optical density (OD) of each well was read at 495 nm by an ELISA reader (BioTek-ELX800, USA). Cultured promastigotes with no drug was used as untreated control and medium with no promastigotes and drugs used as blank. Fifty percent inhibitory concentrations (IC_{50} values) were calculated for all the tested extracts by using Probit test in SPSS software version 24.

2.6. Anti-amastigote inhibitory effect

200 μl of murine macrophages (5×10^5 cells/ml) were added to the 6-chamber plates containing sterile slides, following incubation at 37 °C in 5% CO_2 for 2 h. Then, 200 μl (25×10^5 cells/ml) stationary phase promastigotes were added to macrophages with the ratio of 5:1 for *Leishmania*/macrophage, and incubated in the same condition for 24 h. After this incubation period, free parasites were removed by pipetting fresh medium, and the infected macrophages were treated with 70 μl of methanolic extracts and MA in different concentrations (1, 10, 100 and 1000 μg/mL) at 37 °C and 5% CO_2 for 72 h. Ultimately, treated slides were fixed with methanol, dried, Giemsa stained and precisely examined by using a light microscope. Also, amastigote-infected macrophages with no extracts and macrophages with no parasite and extracts were considered as positive and negative groups, respectively. The anti-amastigote inhibitory effect of the extracts was evaluated by calculating the mean number of amastigotes within each cell of 100 macrophages compared to positive control, in triplicates (Ezatpour et al., 2015). In addition, the IC_{50} values were calculated for all the extracts by the probit test in the SPSS software.

2.7. Flow cytometry

For determination of apoptosis in promastigotes, we used Apoptosis Detection kit of PI Annexin V- fluorescein isothiocyanate (FITC). Cytoplasmic membrane of the apoptotic cell can be structurally altered by exposing phosphatidylserine on their surface, which can be detected by annexin V (Bahrami et al., 2016). For this method, 10^6 promastigotes were seeded in 1.5 mL micro tubes with RPMI-1640 and 10% FBS, then each plant extract or Glucantime ®at 1000 μg/mL concentration were added. We incubated them at 24 °C for 72 h. After this time, promastigotes were centrifuged, washed three times with PBS and resuspended with 1 mL binding buffer. At last, we added annexin V (5 μl) to the reaction and incubated at room temperature (25 °C) in dark condition for 20 min. The results were read by flow cytometry (Becton Dickinson) and analyzed with Cell Quest software. In parallel, settings of untreated and negative control were used.

2.8. Statistical analysis

The IC_{50} and CC_{50} values was calculating by using the SPSS version 24 (SPSS Inc., Chicago, USA). Data analysis was carried out by Prism 7.01 software (GraphPad Software, CA, USA). The differences between extract-treated groups and control group or Glucantime were analyzed by ANOVA. Also, *t*-test was used to analyze the difference between plant extracts. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cytotoxicity

In vitro susceptibility assay displayed no significant cytotoxicity effect in our extracts, as the selectivity index (SI) was 16.2, 7.67, 7.12 and 1.01 for *Q. velutina*, *C. procera*, *N. tabacum* and Glucantime®, respectively (Table 1). SI ≥10 was considered nontoxic. As shown in Fig. 1, *Q. velutina* represented the highest viability of J774 cells in various concentrations compared to the other extracts and Glucantime®. Besides, Glucantime® caused significant lower viability of J774 cells in comparison to three plant extracts, especially in the last four concentrations ($P < 0.001$).

3.2. Anti-promastigote role assessment

The findings demonstrated that methanolic extracts displayed effective antileishmanial activities with a dose-response effect against the promastigote stage of L. *tropica* at all concentrations used; particularly *Q. velutina* showed the lowest viability of promastogotes at concentrations of 100 and 1000 μg/mL ($P < 0.001$) (Fig. 2). The OD values at different concentrations (10, 100 and 1000 μg/mL) of the three medicinal extracts were significantly lower than the corresponding untreated control groups ($P < 0.001$). All extracts exhibited similar pattern of action in reducing tetrazolium; although, Glucantime® exerted more profound activity compared with the control group. The IC_{50} value for *Q. velutina* was 132.6 μg/mL, followed by *C. procera* 248.1 μg/mL and *N. tabacum* 571.2 μg/mL against promastigotes, whereas this value was 588.3 μg/mL for Glucantime® (Table 1). As shown, the IC_{50} value of *Q. velutina* and *C. procera* represented significant lower IC_{50} compared to Glucantime® as control drug ($P < 0.001$), but *N. tabacum* did not. The minimum inhibitory concentration (MIC) for both extracts and Glucantime® was 10 μg/mL (the least dose inhibited the growth rates of L. *tropica* stages, in the present investigation).

3.3. Anti-amastigote role assessment

The results showed that different concentrations of three methanolic extracts and Glucantime® decreased the growth rate of amastigotes (clinical stage). Our plant extracts demonstrated significant reduction in the mean number of amastigotes in cells compared to untreated control at all concentrations, except at 1 μg/mL ($P \leq 0.01$). Besides, *Q. velutina* represented more reducing effect on the amastigotes in comparison to the other extracts and Glucantim®e, especially at 1000 μg/mL concentrations ($P \leq 0.01$) (Fig. 3, Table 2). Also, the IC_{50} value for *Q. velutina* was 51.2 μg/mL, followed by *C. procera* 95.2 μg/mL and *N. tabacum* 118.3 μg/mL against amastigotes, whereas this value was 236.3 μg/mL for Glucantime® as control drug (Table 1). All extracts showed significant reduction in the IC_{50} value of amastigotes compared to Glucantime® ($P \leq 0.001$), as *Q. velutina* showed the lowest one.

3.4. Apoptosis analysis

The results showed that the medicinal extracts displayed different PCD effect on promastigotes of L. *tropica*. The percent of apoptosis at 1000 μg/mL for *Q. velutiona*, *C.procera*, *N. tabacum* and Glucantime® were 37.4%, 18.6%, 8.5% and 52.4%, respectively. Glucantime® induced more apoptotic effect than the plant extracts. Also, *Q. velutiona* represented higher apoptotic effect compared to other extracts (Fig. 4). The apoptotic value of residual methanol used in the plant extracts was negligible (2.3%). Interestingly, the PCD was conversely incremented by increasing the concentration of Glucantime® (Table 3).

4. Discussion

To our knowledge, this is the first investigation on the leishmanicidal effects of these three plant extracts. In the present study the methanolic extracts demonstrated a good index of antileishmanial activity against both stages of L. *tropica* as they proved to be effective in inhibiting the replication rate of promastigotes and intra-macrophage amastigotes. The MIC was 1000 μg/ml for the extracts and also Glucantime® which expressed a similar inhibitory action when the overall potential strength is being considered. Additionally, this leishmanicidal activity could be confirmed by the low OD or IC_{50} values.

Recent global data indicate that leishmaniasis has become a major medical and veterinary health concern as documented in many endemic countries (*WHO technical report series.*, 2010; Alvar et al., 2012). Control of this disease is complicated because of the presence of numerous species of vectors and reservoirs (Ashford, 2000). Treatment of various clinical and epidemiological forms with pentavalent antimonial agents, as the first- standard drugs, over the past 70 years, is unsatisfactory. The choice of so many second-line compounds alone or in combination is also very limited. At present, there is no vaccine available (Noazin et al.,

Table 1
IC_{50} values of *Quercus velutina*, *Calotropis procera* and *Nicotiana tabacum* against the growth rate of promastigotes and amastigotes of *Leishmania tropica* compared with Glucantime® as positive control and the CC_{50} values of drugs on macrophage using the SI index. Data are expressed as the mean ± SD.

Extracts	Amastigote		Promastigote		Macrophage	[c]SI
	[a]IC_{50} ± SD (μg/mL)	*P*-value	[a]IC_{50} ± SD (μg/mL)	*P*-value	[b]CC_{50} (μg/mL)	(Selectivity Index)
Glucantime®	236.3 ± 0.1	NR	588.3 ± 0.1	NR	240.6 ± 0. 3	1.01
Quercus velutina	51.2 ± 0.1	$P < 0.001$	132.6 ± 0.2	$P < 0.001$	830.3 ± 0. 3	16.2
Calotropis procera	95.2 ± 0.1	$P < 0.001$	248.1 ± 0.2	$P < 0.001$	730.6 ± 0. 7	7.67
Nicotiana tabacum	118.3 ± 0.1	$P < 0.001$	571.2 ± 0.1	$P > 0.05$	843.4 ± 0. 3	7.12

NR: Not related.
[a] IC_{50}, Concentration of drug that inhibited 50% of growth in promastigotes and amastigotes.
[b] CC_{50}, Concentration of drug that inhibited 50% of growth in macrophages.
[c] SI, Selectivity index (CC_{50}/IC_{50}).

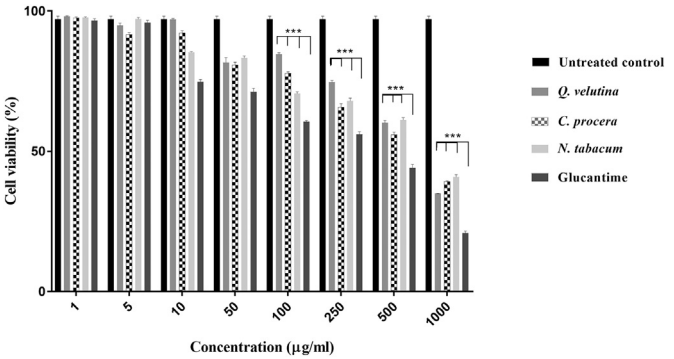

Fig. 1. Cell viability of different concentrations of *Quercus velutina*, *Calotropis procera* and *Nicotiana tabacum* against murine macrophages (J774 cells) in comparison with Glucantime® and untreated control (***$P < 0.001$).

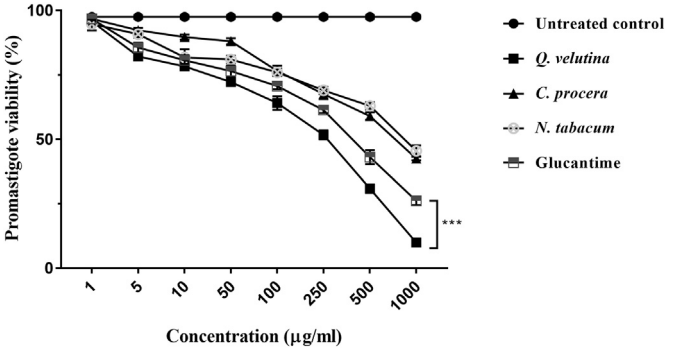

Fig. 2. The viability percent of *Quercus velutina*, *Calotropis procera*, and *Nicotiana tabacum* against *Leishmania tropica* promastigotes compared to Glucantime (***$P < 0.001$).

2008). Therefore, current efforts have been focused on discovering and use of new therapeutic natural agents as they provide rich and potential source of bioactive antileishmanial products.

Natural products, such as medicinal extracts, as crude compounds or pure standardized extracts could provide a valuable source for new medicines due to availability of bioactive compounds, accessibility, safety and low costs (Cos et al., 2006). According to the WHO, approximately 80% of the world's population somehow relies on traditional remedies for their primary healthcare needs. The use of herbal medicines represents a long history of human interactions with the surrounding environment across the globe. Plants used in traditional medicine comprise a wide range of potential compositions that can be essential to treat various disease syndromes caused by infectious agents (Rocha et al., 2005).

The apoptotic program is evolved to regulate cell growth and development in kinetoplastids including *Leishmania* species similar to other eukaryotes (Doroodgar et al., 2016; Bahrami et al., 2016). Programmed cell death is an essential effector mechanism which can play a central role to trigger innate and adaptive cell responses against the parasites (de Freitas Balanco et al., 2001). Further elucidation of the PCD in *Leishmania* could provide valuable information on pathogenesis and biological processing which

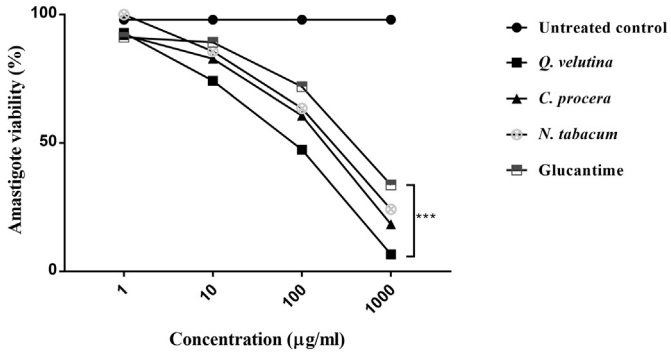

Fig. 3. The viability percent of *Quercus velutina*, *Calotropis procera* and *Nicotiana tabacum* against *Leishmania tropica* amastigotes compared to Glucantime® (***$P < 0.001$).

Table 2
The effect of various concentrations of *Quercus velutina*, *Calotropis procera*, *Nicotiana tabacum* extracts and Glucantime® on the mean number of intramacrophage amastigotes of *Leishmania tropica* in each macrophage.

Concentration (µg/mL)	*Q. velutina*		*C. procera*		*N. tabacum*		Glucantime®	
	Mean ± SD	*P* value	Mean ± SD	*P* value	Mean ± SD	*P* value	Mean ± SD	*P* value
Control	96.6 ± 0.4	NR	96.4 ± 0.4	NR	96.4 ± 0.4	NR	96.4 ± 0.4	NR
1	89.6 ± 0.6	$P > 0.05$	88.8 ± 0.4	$P > 0.05$	98.7 ± 1.1	$P > 0.05$	88.0 ± 0.6	$P > 0.05$
10	71.6 ± 0.6	$P \leq 0.01$	79.8 ± 0.4	$P \leq 0.01$	80.7 ± 1.1	$P > 0.05$	86.0 ± 0.6	$P > 0.05$
100	56.3 ± 0.3	$P \leq 0.01$	58.4 ± 0.4	$P \leq 0.01$	59.3 ± 0.4	$P \leq 0.01$	59.7 ± 0.4	$P \leq 0.01$
1000	4.5 ± 0.3	$P \leq 0.001$	15.8 ± 0.1	$P \leq 0.001$	16.0 ± 0.5	$P \leq 0.001$	32.5 ± 0.1	$P \leq 0.001$

NR: Not related.

might be used to target new effective drugs for growth inhibition. Moreover, it was previously suggested that L. *tropica* promastigotes uniformly express phosphatidylserine in their stationary phase (Tripathi and Gupta, 2003). As revealed the leishmanicidal effects of the above plant extracts was in part mediated through PCD as documented by externalization of PS.

Over the past decades extensive efforts have been made to develop new drug alternatives in endemic countries. In general, a wide range of antileishmanial plant extracts have been evaluated as evidenced from Iran (Bahmani et al., 2015), French Guiana (Rocha et al., 2005), India (Samuelsson and Bohlin, 2017), Israel (El-On, 2009) and Greece (Fokialakis et al., 2007). There seem new approaches toward the natural products, in absence of effective agents would be a logical strategy (Harvey et al., 2015). Unfortunately; although, despite the use of natural products in endemic countries only a limited effort has been made to isolate and analyze their chemical antileishmanial compositions to conduct clinical trials.

5. Conclusion

The findings of this study demonstrate effective antileishmanial activities for the three crude extracts that can be used as a natural source for production of new leishmanicidal agents against CL. However, further experimental and clinical studies are required to isolate and analyze their chemical compositions for final evaluation of biological activities of *Q. velutina, C. procera, and N. tabacum* in animal models as well as in human volunteers as new therapeutic agents.

Financial Support

This investigation was supported by the Leishmaniasis Research Center and Vice Chancellor for Research, Kerman University of Medical Sciences (protocol no. 94/228). The funder had no role in the study design, data collection, data analysis and manuscript preparation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests

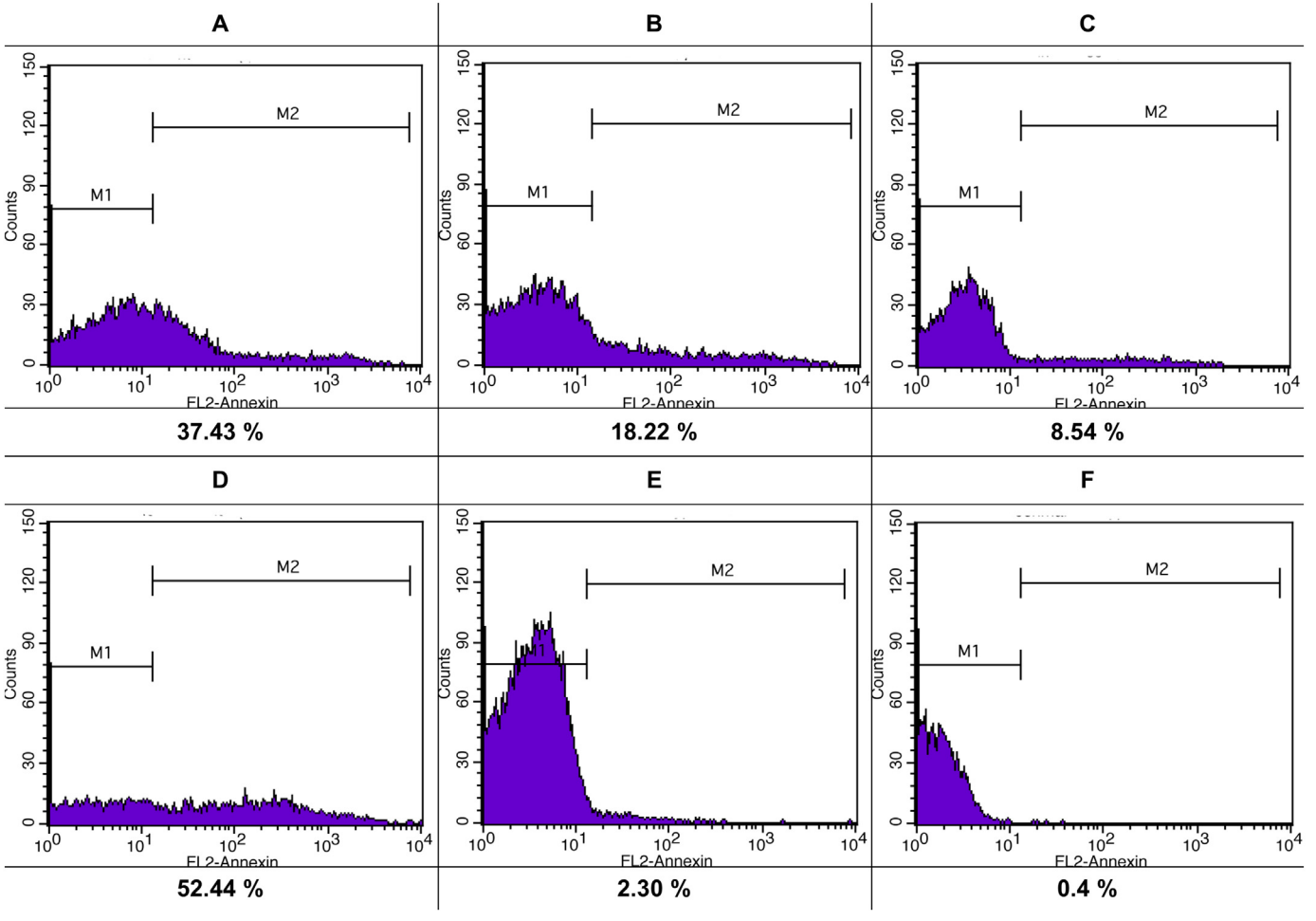

Fig. 4. Apoptotic profiles of *Leishmania tropica* promastigotes with annexin V at 1000 μg/ml of *Q. velutina* (A), *C. procera* (B), *N. tabacum* (C), Glucantime® (D), methanol (E) and negative control (F). M_1 is a region that the organisms show no apoptosis and M_2 showing apoptotic organisms.

Table 3
Apoptosis percentage of *Q. velutiona*, *C. procera*, *N. tabacum* and Glucantime® by flow cytometry analysis.

Treatment	Concentration (μg/ml)	Apoptosis (%)
Quercus velutina	1000	37.4
Calotropis procera	1000	18.6
Nicotina tobacco	1000	8.5
Glucantime®	1000	52.4
Glucantime®	100	12.5
Glucantime	10	9.3
Methanol	NR	2.3
control	NR	0.4

NR: Not related.

Acknowledgments

The authors would like to appreciate all of the staff of Leishmaniasis Research Center for their immense assistance in performing this research.

References

Alvar, J., Vélez, I.D., Bern, C., Herrero, M., Desjeux, P., Cano, J., et al., 2012. Leishmaniasis worldwide and global estimates of its incidence. PLoS One 7, e35671.
Ashford, R.W., 2000. The leishmaniases as emerging and reemerging zoonoses. Int. J. Parasitol. 30, 1269–1281.
Azwanida, N.N., 2015. A review on the extraction methods use in medicinal plants, principle, strength and limitation. Med Aromat Plants. 4, 412–2167.
Bahmani, M., Saki, K., Ezatpour, B., Shahsavari, S., Eftekhari, Z., Jelodari, M., et al., 2015. Leishmaniosis phytotherapy: review of plants used in Iranian traditional medicine on leishmaniasis. Asian Pac. J. Trop. Biomed. 5, 695–701.
Bahrami, S., Khademvatan, S., Razi Jalali, M.H., Pourbaram, S., 2016. Amiodarone triggers induction of apoptosis in cutaneous leishmaniasis agents. Pathogens and global health. 110, 200–204.
Bajaj, S., Wakode, S.R., Khan, W., Manchanda, S., Kumar, S., 2018. Simultaneous HPTLC analysis and in vitro antileishmanic activity of various secondary metabolites in extract of the traditional medicinal herb Artabotrys hexapetalus (lf). Ayu. 39, 92.
Barati, M., Sharifi, I., Sharififar, F., Hakimi Parizi, M., Shokri, A., 2014. Anti-leishmanial activity of gossypium hirsutum L., Ferula Assa-foetida L. and Artemisia aucheri Boiss. Extracts by colorimetric assay. Anti-Infective Agents. 12, 159–164.
Bruchhaus, I., Roeder, T., Rennenberg, A., Heussler, V.T., 2007. Protozoan parasites: programmed cell death as a mechanism of parasitism. Trends Parasitol. 23, 376–383.
Cos, P., Vlietinck, A.J., Vanden, Berghe D., Maes, L., 2006. Anti-infective potential of natural products: how to develop a stronger in vitro 'proof-of-concept. J. Ethnopharmacol. 106, 290–302.
Croft, S.L., Seifert, K., Yardley, V., 2006. Current scenario of drug development for leishmaniasis. Indian J. Med. Res. 123, 399–410.
de Freitas Balanco, J.M., Moreira, M.E.C., Bonomo, A., Bozza, P.T., Amarante-Mendes, G., Pirmez, C., et al., 2001. Apoptotic mimicry by an obligate intracellular parasite downregulates macrophage microbicidal activity. Curr. Biol. 11, 1870–1873.
Doroodgar, M., Delavari, M., Doroodgar, M., Abbasi, A., Taherian, A.A., Doroodgar, A., 2016. Tamoxifen induces apoptosis of Leishmania major promastigotes in vitro. The Korean journal of parasitology. 54, 9.
El-On, J., 2009. Current status and perspectives of the immunotherapy of leishmaniasis. The Israel Medical Association Journal: IMAJ. 11, 623.
Ezatpour, B., Saedi Dezaki, E., Mahmoudvand, H., Azadpour, M., Ezzatkhah, F., 2015. In vitro and in vivo antileishmanial effects of Pistacia khinjuk against Leishmania tropica and Leishmania major. Evid. Based Complement. Alternat. Med. 2015.
Fokialakis, N., Kalpoutzakis, E., Tekwani, B.L., Khan, S.I., Kobaisy, M., Skaltsounis, A.L., et al., 2007. Evaluation of the antimalarial and antileishmanial activity of plants from the Greek island of Crete. J. Nat. Med. 61, 38–45.
Harvey, A.L., Edrada-Ebel, R., Quinn, R.J., 2015. The re-emergence of natural products for drug discovery in the genomics era. Nat. Rev. Drug Discov. 14, 111–129.
Lamidi, M., DiGiorgio, C., Delmas, F., Favel, A., Mve-Mba, C.E., Rondi, M.L., et al., 2005. In vitro cytotoxic, antileishmanial and antifungal activities of ethnopharmacologically selected Gabonese plants. J. Ethnopharmacol. 102, 185–190.
Mahmoudvand, H., Ezzatkhah, F., Sharififar, F., Sharifi, I., Dezaki, E.S., 2015. Antileishmanial and cytotoxic effects of essential oil and methanolic extract of Myrtus communis L. The Korean journal of parasitology. 53, 21.
Moein, M., Hatam, G., Taghavi-Moghadam, R., Zarshenas, M.M., 2017. Antileishmanial activities of Greek Juniper (*Juniperus excelsa* M Bieb.) against leishmania major promastigotes. Journal of evidence-based complementary & alternative medicine 22, 31–36.
Noazin, S., Modabber, F., Khamesipour, A., Smith, P.G., Moulton, L.H., Nasseri, K., et al., 2008. First generation leishmaniasis vaccines: a review of field efficacy trials. Vaccine. 26, 6759–6767.
Rocha, L.G., Almeida, J., Macedo, R.O., Barbosa-Filho, J.M., 2005. A review of natural products with antileishmanial activity. Phytomedicine. 12, 514–535.
Rodrigues JCF, Seabra SH, de Souza W. Apoptosis-like death in parasitic protozoa. Journal of Morphological Sciences. 2017;23: 0.
Samuelsson, G., Bohlin, L., 2017. Drugs of Natural Origin: A Treatise of Pharmacognosy. CRC Press Inc.
Schulz, V., Hänsel, R., Tyler, V.E., 2001. Rational Phytotherapy: A physician's Guide to Herbal Medicine. Psychology Press.
Shokri, A., Sharifi, I., Khamesipour, A., Nakhaee, N., Harandi, M.F., Nosratabadi, J., et al., 2012. The effect of verapamil on in vitro susceptibility of promastigote and amastigote stages of Leishmania tropica to meglumine antimoniate. Parasitol. Res. 110, 1113–1117.
Tripathi, A., Gupta, C.M., 2003. Transbilayer translocation of membrane phosphatidylserine and its role in macrophage invasion in Leishmania promastigotes. Mol. Biochem. Parasitol. 128, 1–9.
World Health Organization (WHO). Report of a meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, Switzerland, 22-26. WHO technical report series. 2010.