Antileishmanial and lung adenocarcinoma cell toxicity of *Withania somnifera* (Linn.) dunal root and fruit extracts

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**ABSTRACT**

This study aims to evaluate the anti-*Leishmania major* and the lung adenocarcinoma (A549) cytotoxicity of *Withania somnifera* root and fruit. The total extracts were obtained by homogenisation in aqueous MeOH, and the sub-extracts [n-hexane, ethyl acetate (EtOAc), n-butanol (n-BuOH), and methanol (MeOH)] were obtained by flash chromatography. The activity evaluation showed that n-BuOH sub-extracts from root and fruit exhibited noticeable antileishmanial promastigote properties. The n-hexane and EtOAc sub-extracts from both organs, and the MeOH sub-extract from the fruit exerted mild to moderate effects on the promastigotes. *In-vitro* growth-inhibitory test results on axenic amastigote and cytotoxicity testing on macrophages (RAW264.7), the parasite-host at the amastigote stage, revealed that the activity was mainly concentrated in the root EtOAc and n-BuOH sub-extracts and to a lesser extent the fruit MeOH and EtOAc, and the root n-hexane sub-extracts. Only the roots’ EtOAc and n-BuOH sub-extracts demonstrated low cytotoxicity on the A549 cell line.

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1. Introduction

Cutaneous leishmaniasis, caused by *Leishmania major*, is an annoying and disfiguring disease (O’Keeffe et al. 2019). Its transmission is through the bite of a female sandfly (*Phlebotomus* genus) and affects around 1,500,000 individuals per year worldwide (Zur 2019). Most of the incidents are in Afghanistan, Algeria, Brazil, Iran, Pakistan, Peru, Colombia and Saudi Arabia (Alvar et al. 2012). Currently, available medicines including pentavalent antimonials, miltefosine, amphotericin B, and antifungal medications are either inadequate in selectivity, highly toxic, or pricey (Ponte-Sucre et al. 2017).

Lung cancer is one of the world’s leading cancer-related causes of mortality (Barta et al. 2019). Widespread use of tobacco products is widely accepted as the leading cause of lung cancer, with a very low relative survival rate, especially in the Middle East region, necessitating intensified efforts to find an urgent remedy.

In this worry, the impulse to exploit conventional herbal drugs for the discovery of alternative therapeutic agents became a priority among researchers. *Withania somnifera* (Linn.) Dunal (Solanaceae), also known as Ashwagandha, Indian ginseng, is a perennial shrub commonly distributed in the Mediterranean region up to South-East Asia in deserts and open fields (Ng et al. 2020). *W. somnifera* is one of the Ayurvedic plants with multiple health benefits. As a folklore cure, it is a well-known botanical method used to improve strength and endurance (Tandon and Yadav 2020). Recent studies have also identified it as a vitality booster, improving homeostasis, tissue nutrition, increasing stress tolerance, aphrodisiac, avoiding degeneration, in addition to a wide array of other health benefits (Rasool and Varalakshmi 2006; Raut et al. 2012; Gupta and Singh 2014; Lopresti and Smith 2021). *W. somnifera* extracts have also demonstrated effectiveness against parasite infections including *Trypanosoma* and *L. donovani* (Nibret and Wink. 2011; Chandrasekaran et al. 2017). It also reduced the rate of mice infection by *L. donovani* and induced immunomodulatory activity (Chandrasekaran et al. 2017).

The biological activity of *W. somnifera* has been attributed primarily to the withanolides [withaferin A, withanolides A–Y, and withanone] which are ergosterane-framed of C28 steroidal lactone triterpenoids (Tandon and Yadav 2020).
In this study, the *in vitro* growth-inhibitory activity of *W. somnifera* root and fruit total extract and their flash-chromatography sub-extracts [n-hexane, EtOAc, n-BuOH, and MeOH] against *L. major* (promastigotes and axenic amastigotes forms), and mouse macrophage-like RAW264.7 cells, and human lung adenocarcinoma (A549) cell lines was examined and compared with miltefosine and etoposide as standard drugs. A comparative study by thin-layer chromatography (TLC) of the total extracts and the biologically active EtOAc and n-BuOH sub-extracts of root and fruit is also shown.

2. Results and discussion

Traditionally used medicinal plants are promising sources of drug leads. As a part of our continuous efforts to expand the role of traditional plants to overcome health problems (Orabi et al. 2020), we investigated *W. somnifera* as a possible therapeutic agent for lung cancer and cutaneous leishmaniasis.

In our study, roots and fruits of *W. somnifera*, collected from mature shrubs were air-dried, powdered and extracted by homogenisation in aqueous MeOH. The extracts were then fractionated into n-hexane, EtOAc, n–butanol, and MeOH sub-extracts using flash chromatography. The activity of all extracts and sub-extracts on the *L. major* promastigotes and axenic amastigotes, RAW264.7 and A549 cell lines was then assessed.

2.1. *In vitro* growth-inhibitory activity on *L. major* and mouse macrophages RAW264.7 cells

The colourimetric cell viability assay using the MTT method was used to determine the *in vitro* growth-inhibitory effect of *W. somnifera* root and fruit total extracts and their sub-extracts on *L. major* promastigotes (Orabi et al. 2020) and axenic amastigotes (Callahan et al. 1997; Ephros et al. 1997). The results (Table S1, Figure S1) revealed that all tested samples except the root MeOH sub-extract and the fruit MeOH total extract (IC50 > 100 µg mL⁻¹) exhibited noticeable anti-*L. major* promastigote activity. The EtOAc sub-extract of the root and fruit exhibited weak activity (IC50 74.0 ± 9.2 and 81.4 ± 3.1 µg mL⁻¹, respectively). The n-BuOH and MeOH sub-extracts of the fruit exhibited noticeable anti-*L. major* promastigote activity (IC50 60.5 ± 0.9 and 69.6 ± 9.7 µg mL⁻¹).

On the other hand, the fruit MeOH and EtOAc, and the root n-hexane sub-extracts showed mild activity on axenic amastigotes (growth inhibition% value of 24.0 ± 2.9%, 21.3 ± 0.3, 36.8 ± 0.5%, respectively, at concentration 100 µg mL⁻¹). The root n-BuOH and EtOAc sub-extracts had the greatest activity on the promastigote (IC50 40.8 ± 4.2 and 74.0 ± 9.2 µg mL⁻¹, respectively) and axenic amastigote forms of the parasite [growth inhibition % values of 43.2 ± 2.9 and 85.6 ± 3.0%, respectively, at concentration 100 µg mL⁻¹ (equivalent to IC43 100 µg mL⁻¹ and IC50 64.1 ± 1.9 µg mL⁻¹, respectively)].

We next investigated the effect of *W. somnifera* fruit and root extracts on macrophages, the final host cells for survival and proliferation of amastigote forms of the *Leishmania* parasites. For this purpose, untreated mouse-macrophage like RAW264.7 cells, and mature macrophages, differentiated from RAW264.7 cells by treatment with lipopolysaccharide (LPS), were employed (Murakami et al. 2020). The results (Figure S2) demonstrated that the major activity against unstimulated macrophages was concentrated into the root n-hexane, EtOAc and n-BuOH extractable metabolites.
Cytotoxicity of these sub-extracts were increased during maturation of the macro-phages (CC$_{50}$ = 46.5 vs 32.7, > 47.6 vs 36.8, and 49.5 vs 27.2 µg mL$^{-1}$). In a previous report, *W. somnifera* polar leaf extracts exhibited macrophages cytotoxicity, at almost comparable value (CC$_{40}$ = 50 and 75 µg mL$^{-1}$) (Chandrasekaran et al. 2013; Kaur et al. 2014) as that shown in our assay by the roots’ polar EtOAc and n-BuOH sub-extracts (Figure S3). Those polar extracts at 15 and 10 µg mL$^{-1}$ have shown a reduction in the mouse macrophages intracellular parasite load by ~50% and ~60%, respectively (Chandrasekaran et al. 2013; Kaur et al. 2014), suggesting a similar mode of activity of our active sub-extracts on the intracellular parasite.

In general, the EtOAc and n-BuOH sub-extracts from roots and fruits were the most promising when analysing the activity against promastigotes and axenic amastigote, but in the aspect of cytotoxicity in macrophages, extracts from roots were more toxic.

There are several processes to achieve the *Leishmania* infection, including the introduction of promastigote in the body, blood circulation of amastigote, phagocytosis and proliferation in the macrophage cell, lysis and circulation again in the blood flow as amastigote (Walker et al. 2014). Therefore, the root n-BuOH and EtOAc sub-extracts may have dual functions; inhibit the promastigote form by the n-BuOH sub-extract, then the EtOAc sub-extract inhibits the axenic amastigote before capturing and after releasing from the cells. The use of EtOAc and butanol fractions in combination may thus have a synergistic effect on the treatment of leishmaniasis.

These findings are in accord with the previously reported results by other research groups, where the antiparasitic activity of *W. somnifera* on *Trypanosoma brucei* has been also shown (Nibret and Wink. 2011). Also, the polar fraction of an alcoholic extract of *W. somnifera* leaves exhibited higher anti- *L. donovani* activity (Chandrasekaran et al. 2013; Kaur et al. 2014), which was ascribed to withanolides content of these fractions (Chandrasekaran et al. 2017).

Although the exact molecules responsible for the activity and their mode of action were not determined in our study, a TLC investigation demonstrated relevance among metabolites of the active anti-leishmanial EtOAc and n-BuOH sub-extracts of the root and fruit (Figure S3). The presence of little qualitative and/or quantitative differences among metabolites of both sub-extracts, may explain the activity variation presented in Table S1 and Figures S1 and S2.

Previous metabolites profiling revealed the presence of withanolides as the main phytoconstituents in *W. somnifera* hydroalcoholic extract of leaf, fruit, and root (Chatterjee et al. 2010; Bolledulla et al. 2012; Trivedi et al. 2017). A previous mechanistic study revealed that withanolides from the leaves of *W. somnifera* were able to induce apoptosis-like death in the promastigote stage of *L. donovani*, reduces parasite count in mouse visceral organs, and modulates host immunity (Grover et al. 2012; Chandrasekaran et al. 2017).

As a result of the anti-*L. major* and macrophages cytotoxicity screening presented in this communication, the significance of this ancient plant as a cheap, sustainable source of antileishmanial drug candidates is growing. The lack of activity assay against amastigote forms of *L. major* internalised in macrophages, as well as a study using isolated compounds, are study’s limitations.
2.2. Growth-inhibitory activity on A549 cell line

We tested the cytotoxic activity of *W. somnifera* root and fruit extracts and sub-extracts on the A549 cell line in our research’ last experiment. As shown in Table S1, only the EtOAc and *n*-BuOH sub-extracts of the root exhibited weak cytotoxic effects on the A549 cell line (IC$_{50}$ 94.8 ± 2.5 and 81.6 ± 4.0 µg mL$^{-1}$, respectively). According to data reported by other research groups, withaferin A, a widely studied bioactive compound from *W. somnifera*, has been shown to have multifunctional antitumor properties in a variety of *in vitro* and *in vivo* cancer models (Dom et al. 2020). Also, withanolide D could effectively induce dose and time-dependent apoptosis in both myeloid (K562) and lymphoid (MOLT-4) cell lines, suppress tumour cell growth in K562 xenograft, but it was non-toxic to normal lymphocytes (Mondal et al. 2010). Higher permissible concentrations from the roots’ EtOAc and *n*-BuOH sub-extracts may have stronger *in vivo* lung anticancer effects, where the extracts’ anti-inflammatory, antioxidant, and immunomodulatory activities (Mehrotra et al. 2011; Rai et al. 2016; Sikandan et al. 2018) may advance its anticancer properties.

3. Conclusion

In our attempt to exploit traditional plant to expand their role in overcoming health problems, the root *n*-BuOH and EtOAc sub-extracts were found promising candidates for the development of antileishmanial drugs. They may inhibit the multiple processes during the lifecycle of the *Leishmania* parasite. The same sub-extracts were mildly cytotoxic to the A549 cell line. It is still necessary to investigate the *in vivo* anti-leishmanial activity and mechanism of action of the active sub-extracts’ metabolites. Also, thorough research into the healing properties of nanoparticles generated from the root most active sub-extracts in a topical dosage form warrants attention in our future work.

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