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Short Communication

White jute (*Corchorus capsularis* L.) leaf extract has potent leishmanicidal activity against *Leishmania donovani*

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ABSTRACT

In pursuit of effective, safe and affordable antileishmanial drugs, the current study was designed to explore *Corchorus capsularis* L. leaf extract (CCEx) as an effective leishmanicidal substitute against *Leishmania donovani*. The leaf extract displays potent antileishmanial activity against *L. donovani* promastigotes with an IC_{50} value of 79.00 ± 0.3 μg/ml. CCEx also significantly induces intracellular reactive oxygen species (ROS) with a concomitant decrease in the level of non-protein thiols in virulent parasites. Additionally, CCEx treatment induces substantial morphological alterations in parasites. Moreover, reagent-based phytochemical analysis of the extract revealed the presence of various phytochemical constituents. Further study is underway to identify the bioactive component(s) or fraction(s) of CCEx through bioassay-guided fractionation.

1. Introduction

Leishmaniasis represents a cluster of diseases with a wide spectrum of clinical manifestations caused by various species of protozoan parasites belonging to the genus *Leishmania*, which is a member of the order *Kinetoplastida* [1]. Throughout the world, nearly 12 million people are affected by leishmaniasis [2]. Leishmaniasis is clinically divided into three main forms, of which visceral leishmaniasis (VL) or kala-azar caused by *L. donovani* is the most prevalent form. The incidences of VL comprise > 50,000 deaths annually, and > 90% of reported cases occur in India, Bangladesh, Nepal, Sudan, and Brazil [3].

In terms of treatment, leishmanial disease is still very challenging due to a lack of appropriate vaccines, and clinically available drugs are also ineffectual at ameliorating the disease. Thus, innovation of novel, less toxic, efficient and economically feasible antileishmanial agents is necessary to replace currently applied drugs. All over the world, natural elements are believed to be harmless and frequently used as traditional therapies against various diseases. Therefore, researchers have also switched their awareness to the plant kingdom in search of new alternative therapies against leishmaniasis with a sustainable cost, as the disease predominantly affects poor communities.

Corchorus capsularis L., commonly called white jute, is widespread with numerous medicinal properties [4]. In various regions of the world, young leaves of *C. capsularis* L. are consumed as healthy vegetables. Furthermore, leaves are also used productively in Ayurvedics for the treatment of fever, liver disorders, ascites, algesia, piles and tumours [5]. Previously, it was reported that the chloroform extract of *C. capsularis* L. leaves exhibits significant anti-nociceptive activity and inflammatory properties [6]. More interestingly, *C. capsularis* L. leaf extract possesses antimicrobial properties [6], but no such study of the leaf extract has been conducted on *Leishmania* spp. Therefore, fuelled by this research deficit, the current study of the antimicrobial properties of the *C. capsularis* L. leaf extract against the *Leishmania* parasite was undertaken.

2. Materials and methods

2.1. Collection and identification of plant material

Leaves of *Corchorus capsularis* L. were collected locally and identified by a taxonomist from the Department of Botany, at the University of Kalyani, and labelled as voucher specimen no: PKP-CCL-03/2015. *Corchorus capsularis* L. (common name: white jute) belongs to the *Malvaceae* (*Tiliaceae*) family and *Corchorus* genus.

2.2. Preparation of leaf extract

Corchorus capsularis L. leaf extract (CCEx) was prepared by slightly modifying the method of Zakaria ZA et al., 2007 [6]. Briefly, the shade-dried leaves of *C. capsularis* L. were pulverized into a coarse powder and then extracted thrice in chloroform at a ratio of 1:20 (w/v) for 72 h. Then, the obtained extract was filtered first through fresh cotton and

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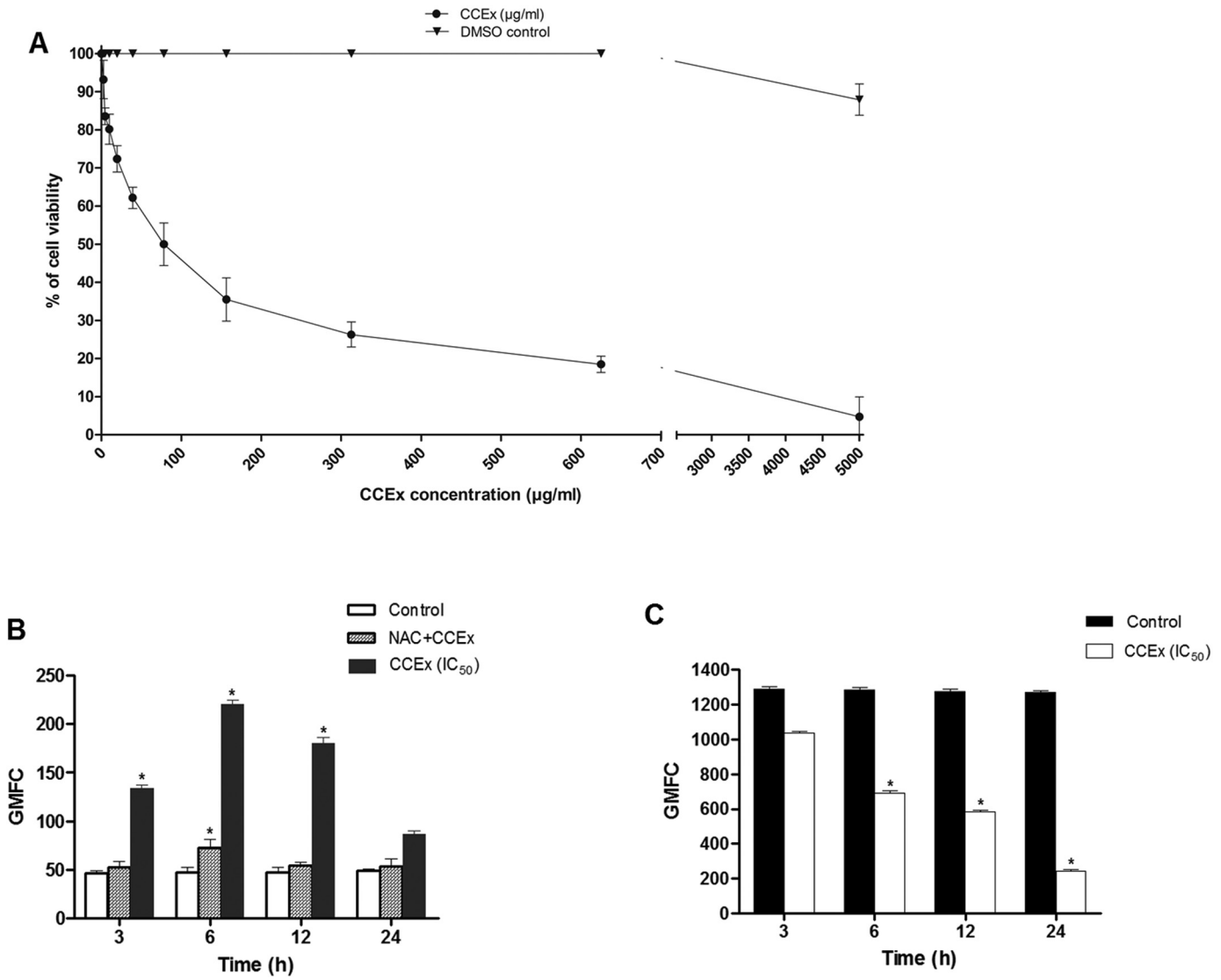

Fig. 1. Antileishmanial activity of CCEx on *L. donovani* promastigotes. (A)Determination of antipromastigote efficacy. Promastigotes (1×10^6/ml) were treated with CCEx (0–5 mg/ml) for 48 h, and an MTT assay was performed. (B)Flow cytometric measurement of intracellular ROS generation. Determination of ROS production was performed in control, CCEx (IC_{50} dose)-treated and NAC (20 mM) pre-treated promastigotes at different time points, i.e., 3 h, 6 h, 12 h, and 24 h. (C)Measurement of intracellular non-protein thiols by flow cytometry. Non-protein thiols were measured in CCEx (IC_{50} dose)-treated and control promastigotes at various time points, i.e., 3 h, 6 h, 12 h, and 24 h. The results are shown from three independent experiments, and the data shown here are statistically significant with a *p-value of < 0.05.

finally passed through Whatman No. 1 filter paper. The dark green-coloured filtered supernatant was dried by simple evaporation of the solvent, the percentage yield of the extract was calculated (9.0 ± 0.2%) and finally reconstituted with dimethyl sulfoxide (DMSO) for biological studies.

2.3. Parasite culture

Promastigotes of *L. donovani*, strain MHOM/IN/1983/AG83, were routinely maintained at 22 °C in Medium 199 (Sigma-Aldrich) containing 100 U/ml penicillin (Gibco), 100 mg/ml streptomycin (Gibco) and supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS, Gibco). The cell viability of parasites was assessed by the trypan blue (Sigma-Aldrich) exclusion method [7].

2.4. Antipromastigote assay

Initially, to determine the effect of CCEx on the viability of *L. donovani* promastigotes, the MTT microplate method was performed [8]. Briefly, log-phase *L. donovani* promastigotes (1×10^6/ml) were seeded in 96-well plates (BD falcon) and treated with CCEx (0–5 mg/ml) in triplicate for 48 h. Then, MTT was added to each well, the plate was incubated for 4 h at 37 °C, and the absorbance corresponding to MTT conversion was measured at 570 nm. The results were expressed as the IC_{50} (50% inhibitory concentration) by graphical representation using GraphPad Prism software (version 5).

2.5. Determination of intracellular ROS generation

To monitor ROS levels in CCEx-treated and untreated *L. donovani* promastigotes, the cell permeable fluorogenic dye H_2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate) was used [9]. In brief, promastigotes (1×10^6/ml) were treated with IC_{50} doses of CCEx for up to 24 h at different time periods, such as 3 h, 6 h, 12 h and 24 h. Afterward, the cells were centrifuged, washed with PBS, resuspended in PBS and incubated with H_2DCFDA (20 µM) for 30 min in the dark at room

temperature. Then, the fluorescence of the H_2DCFDA was monitored using a BD FACSCalibur flow cytometer. Data were analysed with the use of the CellQuestPro software and the geometric mean fluorescence channel (GMFC) represented as a bar chart. In the same experiment, the cells were pre-treated with 20 mM of the ROS inhibitor NAC (*N*-acetyl-L-cysteine) (Sigma–Aldrich).

2.6. Measurement of intracellular non-protein thiols

To measure the level of non-protein thiols, log-phase *L. donovani* promastigotes (1×10^6/ml) were treated with CCEx (IC_{50} dose) at various time points similar to the ROS monitoring periods. Then, the cells were centrifuged, and the cell pellets were washed with PBS and incubated with 10 μM 5-chloromethyl fluorescein diacetate (CMFDA) (Molecular Probes) in the dark for 30 min at room temperature. Data acquisition was performed using the BD FACSCalibur flow cytometer. Then, data were analysed using CellQuestPro software and represented as GMFC [10].

2.7. Morphological observations

The cellular morphology of *L. donovani* promastigotes was examined in the presence or absence of CCEx. Briefly, promastigotes (1×10^5 cells/ml) were treated with an IC_{50} dose of CCEx for 24 h and 48 h. Then, slides of treated and untreated parasites were observed under a light microscope (Meiji, ML 2955). Miltefosine (10 μM) was used as a positive control.

2.8. Phytochemical analysis

The preliminary phytochemical analysis of CCEx was performed according to the standard method [11].

2.9. Statistical analysis

Each experiment was performed at least three times, and representative data were analysed by Student's *t*-test. Values with the least significant differences between control and test groups were considered significant at $^*p < 0.05$.

3. Results

3.1. The antipromastigote activity of CCEx

The antipromastigote activity of CCEx was studied by calculating the 50% inhibitory concentration (IC_{50}) of CCEx against *L. donovani* promastigotes using the MTT assay method (Fig. 1A). CCEx antipromastigote assays were performed with doses ranging from 0 to 5 mg/ml, and from the dose-response curve, we found an IC_{50} value of 79.00 ± 0.3 μg/ml.

3.2. The effect of CCEx on the generation of intracellular ROS in promastigotes

The generation of intracellular ROS is a major indication of apoptosis in promastigotes [9,12]. Therefore, we evaluated intracellular ROS generation in CCEx-treated *L. donovani* promastigotes using a fluorescent probe, H_2DCFDA. The green fluorescent product of this probe is regarded as an indication of ROS production inside the cells. GMFCs of the treated cells were compared to GMFCs of control cells at 3 h, 6 h, 12 h and 24 h. Initially, there was a gradual increase in ROS production up to 6 h in CCEx (IC_{50} dose)-treated cells compared to the control cells. However, beyond 6 h of treatment, the generation of ROS significantly declined with increasing time up to 24 h (Fig. 1B). However, in the presence of NAC, decreased levels of ROS were observed in CCEx-treated parasites and were nearly the same as the levels observed in the control parasites.

3.3. Assessment of non-protein thiols in CCEx-treated promastigotes

Thiols play a significant role in parasite viability, and the depletion of non-protein thiols is a chemotherapeutic proposition for antileishmanial drug targets [13]. Therefore, with this issue in mind, we used the effective fluorescent dye CMFDA to measure non-protein thiols flow cytometrically in CCEx (IC_{50} dose)-treated promastigotes. CMFDA is a cell-permeant probe that rapidly binds to non-protein thiols after entering the cell; inside cells, CMDA is hydrolysed by cellular esterases and eventually converts to a fluorescent thioether [14]. Accordingly, the detected fluorescence is directly proportional to the amount of non-protein thiols present inside the cells. A gradual decrease in non-protein thiols was observed in CCEx-treated promastigotes up to 24 h. The GMFC was appreciably decreased in a time-dependent fashion in CCEx-treated parasites compared to the control parasites (Fig. 1C).

3.4. Morphological alterations in CCEx-treated promastigotes

Microscopically, we observed morphological changes in CCEx (IC_{50} dose)-treated promastigotes compared to the untreated (control) and miltefosine-treated promastigotes at both 24 h and 48 h. The photomicrographs captured by phase contrast microscopy showed that untreated parasites were highly motile, healthy and elongated in shape with long flagella after 24 h and 48 h (Fig. 2A, B). On the other hand, miltefosine (10 μM)-treated promastigotes showed atypical morphology with cell shrinkage and loss of flagella compared to the untreated promastigotes at 24 h and 48 h (Fig. 2E, F). As observed in miltefosine-treated parasites, CCEx-treated parasites also experienced similar morphological deformities at the same time periods (Fig. 2C, D).

3.5. Phytochemical constituents of CCEx

The findings of our primary reagent-based phytochemical investigation of CCEx revealed that phytoconstituents such as flavonoids, terpenoids, saponins, tannins and steroids are the most prominent components of the extract, as shown in Table 1.

4. Discussion

The current toxicity, high cost and drug resistance issues of clinically applied drugs against leishmaniasis invoked our interest in exploring new potent antileishmanial agents to protect humanity from leishmaniasis. Recently, several medicinal plants have been investigated for their antiparasitic properties [15]. Leaves of *C. capsularis* L. have multifarious medicinal value, but we are the first to report the leishmanicidal efficacy of the chloroform extract of *C. capsularis* L. leaf against *L. donovani*. Herein, we prepared a chloroform extract of *C. capsularis* L. leaf and explored its in vitro leishmanicidal activity against *L. donovani*. First, we found appreciable inhibitory effects of CCEx against *L. donovani* promastigotes.

Next, we focused on the evaluation of intracellular ROS production upon treatment because the viability of promastigotes gradually declines with increasing ROS levels [12]. Interestingly, CCEx caused an increase in cellular ROS production and displayed an antiparasitic effect. We also confirmed our results of ROS production by using a well-established ROS quencher, NAC. Conversely, reports have revealed that non-protein thiols are molecules that are well-known to protect cells against ROS-mediated oxidative damage [16]. Therefore, our observation of ROS-mediated activity upon CCEx treatment raised the question of whether there is a diminution of non-protein thiols upon treatment with CCEx. A study found that antimony exerted antileishmanial activity followed by the production of ROS and depletion of thiols [17]. Therefore, we monitored the level of total intracellular non-protein thiols at different time points up to 24 h, similar to the ROS monitoring

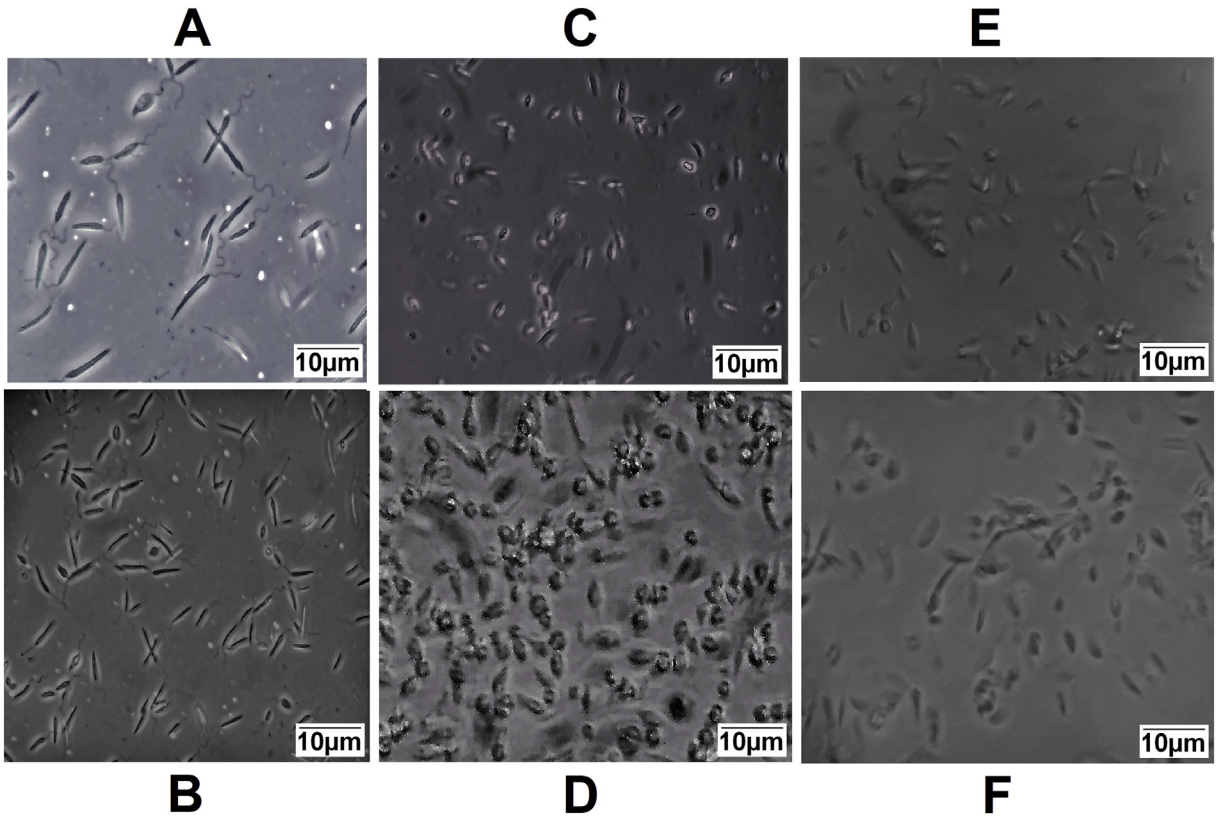

Fig. 2. Morphological alterations in CCEx-treated *L. donovani* promastigotes. Images are presented from three independent experiments. (A, B)Untreated parasites at 24 h and 48 h time points, respectively. (C, D)CCEx-treated parasites at 24 h and 48 h time points, respectively. (E, F)Miltefosine (10 μM)-treated parasites at 24 h and 48 h time points, respectively.

Table 1
Phytochemical constituents of the extract.

Sl. No.	Phytochemical constituents	Methods used	Status
1	Flavonoids	Alkaline reagent test	Present
2	Terpenoids	Salkowski test	Present
3	Saponins	Froth test	Present
4	Tannins	Ferric chloride test	Present
5	Steroids	Liebermann-Burchard's test	Present
6	Alkaloids	Dragendroff's test	Absent

period, and we found that the non-protein thiol level was significantly reduced in the CCEx-treated parasites when compared to the untreated control parasites. Thus, the attenuation of intracellular thiols with enhanced intracellular ROS production in CCEx-treated parasites leads to decreased parasite survival and demonstrates the leishmanicidal nature of the extract. Afterward, by microscopy, we observed a drastic change in parasitic structure in CCEx-treated cells compared to the untreated control cells. This microscopic observation of morphological alterations of the parasites also indicates the leishmanicidal property of CCEx.

The different parts of plants contain a vast number of various secondary metabolites, many of which have defensive properties against bacteria, fungi and other microorganisms [18]. Accordingly, CCEx was investigated for the identification of different types of phytochemical constituents by reagent-based phytochemical analysis. The preliminary findings of the various phytochemical constituents in CCEx were corroborated by data reported in the literature [6]. Collectively, growth inhibition of *L. donovani* promastigotes by CCEx treatment with significant morphological alterations demonstrated the antileishmanial properties of the extract. Simultaneously, the time-dependent augmentation of ROS production with the consequent decline of total intracellular non-protein thiols also highlights the antileishmanial potential of the extract. Furthermore, the existence of different secondary metabolites in CCEx indicates that further investigation is needed to identify and separate any bioactive compound(s). Therefore, the findings of this study might enable the successful development of leishmanicidal compound(s) from CCEx in the very near future for the battle against *Leishmania* infection.

5. Conclusion

From our findings, we can conclude that the chloroform extract of *C. capsularis* L. leaf exhibits significant leishmanicidal activity against *L. donovani*. Moreover, the various phytoconstituents present in the extract may be responsible for the antileishmanial activity. Therefore, further study is required to identify and confirm the extract-derived fraction(s) or molecule(s) liable for leishmanicidal activities.

Conflicts of interest

The authors declare no conflicts of interest.

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