Biosynthesis of anti-leishmanial natural products in callus cultures of *Artiumisia scoparia*

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**ABSTRACT**

Clinically, available synthetic chemotherapeutics in the treatment for leishmaniasis are associated with serious complications, such as toxicity and emergence of resistance. Natural products from plants can provide better remedies against the Leishmania parasite and can possibly minimize the associated side effects. In this study, various extracts of the callus cultures of *Artimisia scoparia* established in response to different plant growth regulators (PGRs) were evaluated for their anti-leishmanial effects against *Leishmania tropica* promastigotes, followed by an investigation of the possible mechanism of action through reactive apoptosis assay using fluorescent microscopy. Amongst the different callus extracts, higher anti-leishmanial activity (IC$_{50}$: 19.13 mg/mL) was observed in the callus raised in-vitro in the presence of 6-Benzylaminopurine (BA) plus 2,4-Dichlorophenoxyacetic Acid (2,4-D) at the concentration of 1.5 mg/L, each. Further, the results of apoptosis assay showed a large number of early-stage apoptotic (EA) and late-stage apoptotic (LA) cells in the *Leishmania* under the effect of callus extract grown in-vitro at BA plus 2,4-D. For the determination of the potent natural products in the callus extracts responsible for the anti-leishmanial activity, extracts were subjected to Gas chromatography-mass spectrometry (GC-MS) for the metabolite analysis. Nonetheless, higher levels of the metabolites, such as nerolidol (22%), pelletierine (18%), aspidin (15%) and ascaridole (11%) were detected in the callus grown in-vitro at BA plus 2,4-D (1.5 mg/L, each). This protocol determines a novel method of production of anti-leishmanial natural products through callus cultures of *A. scoparia*, a medicinal plant.

**Introduction**

Leishmaniasis is a hazardous infectious disease caused by more than 20 different leishmanial parasites and is amongst the most neglected tropical diseases [1]. According to the World Health Organization (WHO), about 12 million people worldwide are suffering from being infected with different leishmanial species [2,3]. In the recent past, an upsurge in leishmaniasis has been witnessed around the globe, especially in the least developed world including Pakistan. The currently available methods in the treatment of leishmaniasis include the use of pentavalent antimonial drugs, such as amphotericin B, pentamidine and paromomycin, those prescribed at first-line for the treatment of leishmaniasis. However, these drugs are associated with many demerits for instance, problems in oral administration, low efficacy, unpleasant side effects, high cost and renal toxicity [4,5]. Further, the parasites may develop resistance to the regular administration of these drugs, which is a risk alert [6]. Therefore, alternative platforms must be explored to develop anti-leishmanial drugs which should be economical, highly effective, easy to access and more human-friendly. Extracts derived from a variety of medicinal plants have been tested against leishmaniasis, for instance, *Allium cepa* (aqueous extract), *Allium sativum* (methanol), *Khaya anthotheca* (hexane, petroleum), *Lantana camara* (oil), *Maesopsis eminii* (dichloromethane), *Morinda citrifolia* (fruit extract), *Porophyllum ruderale* (alkyl extract), *Tylorhora hirsute* (methanol extract), etc., have shown promising anti-leishmanial activities [2]. The ample anti-leishmanial activity shown by the extracts of these plants is due to the presence of bioactive compounds, such as polyphenols, flavonoids, alkaloids, tannins, essential oil, etc. *Artemisia scoparia* is one of the most significant medicinal plant species of the genus *Artemisia* [7]. In Pakistan, it is distributed in the arid and semi-arid areas of Balochistan, KPK, northern Punjab and Kashmir [8]. The extract, from different parts of the plant, has shown multiple biological activities, such as anti-cholesterolemia, antipyretic, antisepsic, antibacterial, chologogue, diuretic, purgative, dilator, and anti-asthenic [9]. *Artemisia scoparia* has also been studied for its potential in treatment against hepatitis, diabetes, jaundice and liver disorders [10]. Besides, its essential oils have shown positive insecticidal and anti-
microbial effects [11]. The phytochemical profile A. scoparia has revealed the presence of biologically active flavonoids, coumarins, essential oils [12–14], scoparone [15], scoparic acid and artemisanolsterol [16]. Biosynthesis of medicinally active metabolites in wild grown medicinal plants is limited and affected by many factors, for instance, geographic variations, particular growth and the developmental stage of the plant, specific season, nutrients availability and environmental contamination [17]. However, to combat the environmental and geographic constraints, plant cell culture technology provides promising means for production of healthy plant material with phytochemically sustainable profiles, in short time and limited space [18]. The callus cultures are the preferred type of plant in vitro cultures, used for the production of healthy biomass under controlled growth conditions from which medicinally potent natural products can be extracted [19]. In many circumstances when compared with the natural plants, callus cultures established in the presence of different plant growth regulators (PGRs) have been found to produce important metabolites in bulk [20]. Nonetheless, there are adequate chances for biosynthesis of some novel metabolites through callus cultures, those not present in the natural plants [21]. Hence, the establishment of callus cultures in A. scoparia might provide an array of medicinally potent metabolites which can act as suitable candidates in the formulation of effective anti-leishmanial drugs.

Material and methods

Establishment of in vitro callus cultures

Wild grown plants of A. scoparia were collected from their natural habitat in the valley of Swat in June 2017. These plants were used to harvest the leaf explants and to develop callus cultures. Leaf pieces were cut to about 3–4 mm² for making explants and were treated with 2.0% sodium hypochlorite with 2–3 drops of Tween 20 (Merck, Kenilworth, NJ) for 20 min. The explants were rinsed five times in sterile distilled water, followed by dipping in a diluted solution of mercuric chloride (HgCl₂, 0.05% w/v) for ten min. Then, they were finally rinsed five times in sterile distilled water. For callus induction, the surface sterilized leaf explants were inoculated on MS media [22], added with the PGRs including BA and 2,4-D either alone at varying levels (0.5, 1.0, 1.5, 2.0 and 2.0 mg/L) or combination of 1.5 mg/L BA plus 0.5, 1.0, 1.5, 2.0 and 2.0 mg/L of 2,4-D. The MS media was supplemented with 30 g/L sucrose and 8 g/L gelling agent (Oxoid, Basingstoke, England). The pH of the media was attuned to 5.8 (Eutech Instruments pH 510, Singapore). The flasks containing media were autoclaved for sterilization at 121°C for 20 min (Systec, Linden, Germany). For control treatment, culture flasks were supplied only with MS media, devoid of PGRs. After inoculation of the explants, the cultured flasks were then placed in the growth chamber maintained at the temperature of 25 ± 1°C under a light intensity of 2000–2500 lux. The photo-period of the chamber was set at 16/8 h for the culture development. After 30 d of the culture period, data was taken as callus induction frequency (percent of responding explants), the day of callus initiation in explants, biomass formation (g/L), and callus colour and callus morphology. For the determination of fresh weight (FW), fine callus from the flasks was collected, washed with sterilized distilled water and then pressed in filter papers (Whatman Ltd., Maidstone, England) to take out excess water. These calli were finally weighed. Similarly, dry weight (DW) was investigated. For which calli were dried at 50°C in the oven (Thermo Scientific, Bremen, Germany) and were weighed. Fresh and DWs were indicated in g/L as per the method of Khan et al. [21].

For proliferation and biomass accumulation, 30-d old calli (3.2 g/L DW) was shifted to fresh MS basal media supplemented with either 1.5 mg I-1BA or 2.0 mg I-1 2,4-D or 1.5 mg I-1BA plus 1.5 mg I-1 2,4-D in Erlenmeyer flask (100 mL). The growth dynamics of the multiplying calli was determined for 45-d period with an interval of 5 d. Data on callus biomass accumulation was recorded as DW (g/L).

Determination of anti-leishmanial activity in callus cell lines

Extract preparation and parasite culture for the assays

Based on the maximum growth, the calli established in response to 1.5 mg/L BA, 2.0 mg/L 2,4-D and 1.5 mg/L BA plus 2.0 mg/L 2,4-D were harvested from the culture flasks and were used for the biological assays. For control treatment, leaf pieces of wild grown A. scoparia were selected for evaluation of the anti-leishmanial activity. The four different extracts used in this study were prepared as previously reported by Ul-Haq et al. [23]. Briefly, the oven-dried callus tissues of the A. scoparia from the selected treatments and wild grown plantlets were ground with mortar and pestle and extracted with methanol. For parasite culture, Leishmania tropica (KWH23) isolates obtained from the Department of Biotechnology, Islamic International University Islamabad, Pakistan, were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated foetal bovine serum (HIFBS), 1% Pen-Strep at 25 ± 1°C (in 25 cm² flasks – TPP® Sigma-Aldrich, St. Louis, MO). After 4 d of incubation, the parasite culture was monitored using an inverted microscope (Olympus®, Tokyo, Japan) and passage for further growth.

Promastigote proliferation measurements by MTT assay

The promastigote forms of L. tropica (1 x 10⁴ cells/well) were seeded in 96-well microtiter plates (Sigma-Aldrich, St. Louis, MO) in RPMI-1640 (Gibco®, Carlsbad, CA) and 10% FBS (Gibco®, Carlsbad, CA) and allowed to grow in the presence of various concentrations (100, 500 and 1000 μg/mL) of each selected A. scoparia extracts or 0.1% DMSO (as negative control) or amphotericin B (as positive controls) for 24, 48 and 72 h at 25 ± 1°C. The anti-leishmanial activity was evaluated using an MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)-based micro-assay as a marker of cell viability according to the protocol of Nadhman et al. [24]. After the incubation period, a 100 μL of MTT solution (5 mg/mL PBS – Sigma Chemical Co., St. Louis, MO) was added to each well and incubated for 4 h at 31°C. The enzymatic reaction was then stopped by the addition of 60 μL DMSO. Relative optical density (OD) was measured at 570 nm using a
Multi-well microtiter plate reader (Bio-Tek ELx-800, Winooski, VT). The absorbance produced by the action of mitochondrial dehydrogenases of metabolically active cells was shown to correlate with the number of viable cells. The assay was performed in triplicate.

**Apoptosis assay**

Apoptosis was analyzed according to the ethidium bromide and acridine orange (EB/AO) staining assay as previously described by Nadhman et al. [24]. Briefly, L. tropica promastigotes were incubated with A. scoparia selected extracts at a final concentration of 100, 500 and 1000 µg/mL and control (0.1% DMSO) for 72 h. The cells were washed with phosphate buffer saline (PBS) by centrifugation (1000 x g, 5 min) and treated with RNase I (1 mg/mL) before staining it with the mixture of EB (100 µg/mL) and AO (100 µg/mL) in a 3:1 concentration. The variance in fluorescence was measured on a Leica fluorescent microscope with a Canon camera using 530 and 485 nm filters for emission and excitation wavelengths, respectively [24].

**Determination of polyphenolic content and anti-oxidant activity**

In order to determine the significant role of plant secondary metabolites in the anti-leishmanial potential of the plant extracts, assays on the determination of polyphenolic content were demonstrated. For extract preparation, the dried callus tissues of each selected sample and control sample were powder by using mortar and pestle. Further, 10 mg of dried powder was mixed in a test tube with solvent ethanol. The mixture was kept for 1 week period in order to get the maximum extract. To remove cells debris, the mixture was centrifuged at 10,000 rpm for 10 min. The supernatant extracted from each sample was collected in fresh tubes and was subsequently used for the investigation of phenolic and flavonoid contents. For both phenolics and flavonoid determination, the protocol of Khan et al. [21] was followed. For extract preparation, the dried powder was mixed in a test tube with solvent ethanol. The mixture was kept for 1 week period in order to get the maximum extract. To remove cells debris, the mixture was centrifuged at 10,000 rpm for 10 min. The supernatant extracted from each sample was collected in fresh tubes and was subsequently used for the investigation of phenolic and flavonoid contents. For both phenolics and flavonoid determination, the protocol of Khan et al. [21] was followed.

**Gas chromatography-mass spectrometry (GC-MS) analysis of the prepared extracts**

Methanol extracts of A. scoparia selected callus and control samples were prepared by cold percolation method. GC-MS analyses were carried out using GC-MS analyzer (Shimadzu GC-MS-QP 2020 system; Shimadzu Corporation, Kyoto, Japan) with autosampler. All the samples were filtered through Whatman (0.2 µm) filter paper. The specifications of the column (RTx5-5) used during the process were configured by the length (50 m), inside diameter (0.25 mm) and film coating (0.25 µm). The carrier gas was helium (99.9%), which was used in a split mode at a flow rate (1 mL/min). Volume of 1 mL of each selected biological sample was injected into the column (280 °C inlet temperature). Setting an initial temperature at 50 °C for 2 min followed by lifting to 300 °C at the rate of 15 °C/min. Ion sources were maintained at 280 °C temperature. For each sample run through MS, the total running time was 30 min with 8 min solvent delay. Spectrum profiles obtained after the process were compared with the online database at the National Institute of Standards and Technology (NIST) 1 or the published reports [9,26].

**Analysis of data**

All the experiments were conducted in triplicates and revised twice. Mean values were determined by using one-way analysis of variance (ANOVA) through Statistix version 8.1 (Analytical Software, Tallahassee, FL) software and were presented with standard errors (±).

**Results and discussion**

The main objectives of this study were to establish callus cultures in A. scoparia for their potential against leishmaniasis and to elucidate the putative anti-leishmanial metabolites in the callus cultures.

**Establishment of callus cultures in Artemisia scoparia**

The in vitro growth potentials of notable cytokinins including BA and auxins, such as 2,4-D and NAA in callus organogenesis of different medicinally important Artemisia plant species have been evaluated by several research groups [20,27,28].

In this study, the effects of BA and 2,4-D at varying levels and combinations were tested on the callus induction frequency, biomass formation and callus morphology in leaf explants of A. scoparia. Within 3-8 d of the culture period, callogenesis was initiated at the cut ends of explants cultured on solid MS basal medium.

When tested alone, BA (1.5 mg/L) resulted in the highest callus induction frequency (63.3%) and biomass formation (FW: 30 ± 1.6 g/L and DW: 8 ± 0.06 g/L). Callus induction in the explants started on the 8th day of the culture period (Table 1). In a similar report, the highest callus growth was...
observed in the leaf explant of *Artemisia absinthium* incubated on MS media supplemented with 2.0 mg/L BA [28]. Within the different concentrations of 2,4-D tested alone, the maximum callus induction frequency (86.5%) was observed at 2.0 mg/L, wherein the fresh biomass 32 ± 1.7 g/L and dry biomass 11 ± 1.0 g/L were recorded in culture flasks. Callus formation in the explants was initiated on the 5th day of the culture period (Table 1). In another study, 0.5 mg/L 2,4-D was found as the most influential PGR on callus induction and biomass formation in leaf explants of *Clinacanthus nutans* [29]. At lower and higher concentrations beyond an optimal level of each PGR negatively affected the callus induction frequency and biomass formation. Screening of the optimal level of any PGR during plant cell culture studies is crucial for optimization of any protocol that can be exploited for industrial production of uniform and healthy biomass of important medicinal plants [27,30,31]. Similar to our results 2,4-D at higher concentrations (>5.0 mg/L) was found to negatively affect the callus growth and biomass formation in commercially important plants [19]. No callus growth was observed in control treatment, wherein explants were cultured on MS media lacking any PGR. Significantly, higher growth response in callus cultures was observed at the combination of BA and 2,4-D, each added at 1.5 mg/L in the MS media. At this treatment, the highest callus induction frequency (89.2%) and maximum biomass formation (FW: 36 ± 1.8 g/L and DW: 14 ± 1.1 g/L) were, respectively, recorded in the cultures. Similarly, higher callus induction frequency (75%) and callus biomass formation were observed in *Cnidium officinale* on MS medium containing 2.3 μM 2,4-D plus 2.2 μM BA [19]. Callus induction and growth are highly influenced by supplementation of the culture media with a combination of auxin and cytokinin at equal concentrations. For instance, Chaaban et al. [30] have observed the highest value of callus biomass production in *Crataegus azarolus* L on MS medium supplemented with 2,4-D plus BA (1.0 mg/L, each). Morphological observations of callus cultures in this study revealed production of green compact, green friable and whitish granular calli on MS media in response to BA, 2,4-D or BA plus 2,4-D treatments, respectively (Table 1; Figure 1). Such culture characteristics in callus cultures as a function of BA and 2,4-D are reported in several other medicinal plants [27,30,31]. However, differences in callus culture characteristics are generally attributed to the type of explant, type and concentration of the PGRs and composition of the growth media used during *in vitro* cultures [25,27,32]. Interestingly, the combination of PGRs, compared with individual levels of each PGR quickly induced callus formation, i.e. on 3rd day in explants following culture inoculation. As the concentration of 2,4-D increased to 2.5 mg/L when combined with 1.5 mg/L BA in the culture media, a decline in the callus growth parameters was observed (Table 1) This is in agreement with Adil et al. [19] who studied the effects of 2,4-D in combination with BA at varying levels on callus growth in *Cnidium officinale*.

Data on callus growth proliferation and dynamics on solid MS media in the presence of 1.5 mg/L BA or 2.0 mg/L 2,4-D

### Table 1. Effects of different plant growth regulators (PGRs) in different levels or combinations on callus growth attributes in *A. scoparia*.

| MS media and PGRs | Concentration (mg/L) | Callus induction frequency (%) | Callus initiation day | Fresh biomass (g/L) | Dry biomass (g/L) | Callus color | Callus morphology |
|------------------|----------------------|-------------------------------|----------------------|---------------------|------------------|--------------|-----------------|
| BA               | 0.5                  | 24.2 ± 1.4                    | Day 8                | 20 ± 1.1            | 4 ± 0.02         | Green        | Compact         |
|                  | 1.0                  | 38.1 ± 2.0                    | Day 8                | 23 ± 1.2            | 5 ± 0.03         | Green        | Compact         |
|                  | 1.5                  | 63.3 ± 3.1                    | Day 8                | 30 ± 1.6            | 8 ± 0.06         | Green        | Compact         |
|                  | 2.0                  | 52.3 ± 2.5                    | Day 8                | 26 ± 1.5            | 7 ± 0.06         | Green        | Compact         |
|                  | 2.5                  | 41 ± 2.2                      | Day 8                | 24 ± 1.4            | 6 ± 0.05         | Green        | Compact         |
| 2,4-D            | 0.5                  | 36 ± 1.8                      | Day 5                | 26 ± 1.5            | 7 ± 0.06         | Green        | Friable         |
|                  | 1.0                  | 58.2 ± 2.8                    | Day 5                | 27 ± 1.5            | 8 ± 0.06         | Green        | Friable         |
|                  | 1.5                  | 69.3 ± 3.3                    | Day 5                | 28 ± 1.6            | 9 ± 0.06         | Green        | Friable         |
|                  | 2.0                  | 86.5 ± 3.5                    | Day 5                | 32 ± 1.7            | 11 ± 1.0         | Green        | Friable         |
|                  | 2.5                  | 51 ± 2.5                      | Day 5                | 24 ± 1.4            | 6 ± 0.05         | Green        | Friable         |
| 2,4-D + BA       | 0.5:1.5              | 44.6 ± 2.4                    | Day 3                | 19 ± 1.1            | 3 ± 0.05         | Whitish yellow | Granular        |
|                  | 1.0:1.5              | 59.1 ± 2.8                    | Day 3                | 31 ± 1.7            | 9 ± 0.08         | Whitish yellow | Granular        |
|                  | 1.5:1.5              | 89.2 ± 3.8                    | Day 3                | 36 ± 1.8            | 14 ± 1.1         | Whitish yellow | Granular        |
|                  | 2.0:1.5              | 62.1 ± 3.1                    | Day 3                | 29 ± 1.6            | 9 ± 0.08         | Whitish yellow | Granular        |
|                  | 2.5:1.5              | 38 ± 2.0                      | Day 3                | 24 ± 1.4            | 6 ± 0.05         | Whitish yellow | Granular        |

Data was collected from three replicates. Means with same alphabets are not significantly different in accordance with DMRT, at $p < 0.05$.}

![Figure 1](image_url). Pictorial presentation of the *in vitro* callus cultures in *A. scoparia*: (a) Wild grown plant, (b) Callus biomass formation in response to BA, (c) Callus biomass formation in response to 2,4-D and (d) Callus biomass formation in response to BA plus 2,4-D.
or 1.5 mg/L BA plus 1.5 mg/L 2,4-D showed significant variations in the accumulation of callus biomass with passage of time (days) in culture period (Figure 2(a–c)). Callus growth dynamics were studied to assess the impacts of these PGRs on dry biomass accumulation in total 45 d of culture period.

It is important to evaluate the callus biomass formation in a temporal measurement of the callus growth during in vitro cultures to decide the time interval inductive for the accumulation of maximum biomass [33].

In this study, at 1.5 mg/L BA the growth curve demonstrated a lag phase of 15 d, log phase of 20 d and a stationary phase of 10 d in the growth cycle (Figure 2(a)). Compared with the initial callus biomass, a gradual and steady growth in callus biomass was observed. Maximum callus biomass accumulation (10.35 g/L DW) was observed on day 36 of the growth curve. However, a significant decrease in the callus biomass accumulation was noticed in the growth curve after 35 d in the stationary phase and the lowest biomass (8.1 g/L DW) was recorded on day 44 in the growth curve. In response to 2.0 mg/L 2,4-D shorter log phase of 10 d and comparatively longer lag and stationary phases of 15 d, respectively, were observed in the growth curve. In the log phase on day 25 of the growth curve, highest biomass (14 g/L DW) was accumulated during callus growth. After day 32, the callus biomass declined subsequently (Figure 2(b)).

Our results are comparable with Huang et al. [33] who observed a significant increase in the biomass during subculture of the *Angelica sinensis* callus on the MS media in the presence of 1.0 mg/L 2,4-D. Further, the callus growth curve established in response to the combination of BA and 2,4-D (1.5 mg/L each), revealed the presence of shorter exponential phase as compared with the lag and stationary phases during callus proliferation. Maximum biomass formation (14 g/L DW) was observed on day 15 and a subsequent decrease in biomass production was observed after day 25 in the growth curve. Wherein, the lowest dry biomass (9 g/L DW) was accumulated on day 40 in the stationary phase. Interestingly when treated with BA plus 2,4-D, the callus cultures resulted in the higher biomass formation on the day 15 of the growth curve as compared with BA and 2,4-D used alone (Figure 2(c)). Though no previous report is available on the callus growth dynamics of *A. scoparia*. However, in the related *Artemisia* species, such as *Artimisia absinthium* L, the callus growth curve was established for 49 d of the culture period. Wherein, maximum callus dry biomass (8.73 g/L) was accumulated on day 42 in MS media containing 1.0 mg/L TDZ plus 1.0 mg/L NAA [32].

**MTT assay for estimation of anti-leishmanial potential in callus extracts**

In this study, the anti-leishmanial potential of established callus cell lines in comparison to the control sample was determined against *L. tropica* promastigotes. Extracts of selected plant samples were tested against leishmaniasis after 72 h incubation and data was taken as percent inhibition of the parasite (Figure 3; Table 2). Results showed that the metabolic activities of the parasite exposed to different doses (100, 500 and 1000 µg/mL) of the selected callus extracts changed with the change in concentration of samples (extracts) as compared to control group. Generally, the formation of purple coloured formazan crystals during MTT assay is the indication of metabolic activity of the parasite,

**Figure 2.** Temporal accumulation of biomass in callus cultures in response to different PGR treatments: (a) 1.5 mg/L BA, (b) 2.0 mg/L 2,4-D and (c) 1.5 mg/L BA plus 1.5 mg/L 2,4-D.
signifying that parasites are alive and sustaining their normal metabolism [2]. In the control sample, dark purple coloured crystals in the assay plate were visually observed. However, in case of exposure to the different extracts of callus samples, *L. tropica* formazan crystals appeared in a very small amount (light purple colour), indicating that the callus cell lines produced a variety of secondary metabolites which stopped the metabolic activities of the *L. tropica* promastigotes. Within the different callus extracts, higher anti-leishmanial activity (IC₅₀ value 19.13 µg/mL) was observed in the callus raised in vitro in the presence of BA plus 2,4-D (1.5 mg/L, each) (Figure 3; Table 2). However, 2,4-D and BA mediated callus extracts showed a moderate activity with IC₅₀ value 263.42 and 474.89 µg/mL, respectively. The control sample showed lesser anti-leishmanial activity with IC₅₀ value 820.00 µg/mL (Table 2). At a higher dose (1000 µg/mL) of each extract, the MTT assay showed similar results in the context of growth inhibition of the parasite by all the tested plant extracts. However, significant variations in the anti-leishmanial activity were observed among the callus extracts at a lower dose (100 µg/mL), tested against *L. tropica* (Figure 3; Table 2). At 1000 µg/mL, all the tested extracts showed a high anti-leishmanial activity with maximum growth inhibition (79.2%) in the callus extracts raised in vitro in combination of BA plus 2,4-D (1.5 mg/L, each) showed the highest activity compared with the callus extracts raised in vitro at BA or 2,4-D separately. The positive control AmB showed 100% activity at all the concentrations tested (results not shown). The higher anti-leishmanial activity demonstrated by the callus extracts of *A. scoparia* can be linked to the higher production of phenolic compounds in the callus culture [34]. Phenolics and flavonoids are the secondary metabolites reportedly known as anti-parasitic drugs [35]. Moreover, plant-derived phenolics and flavonoids have been reported to exhibit anti-leishmanial activity [36] for instance the flavonoids of *A. indica*, showed maximum activity against the leishmanial parasite [37]. The mechanism of action by these compounds is recognized by their ability to interpolate the DNA through obstruction of the DNA enzymes, such as topoisomerase I or II, which are necessary for the process of duplication. If DNA replication stops, topoisomerasers are blocked and thus leishmanial cells cannot multiply [38].

### Table 2. Percentage inhibition (IC₅₀) values of *L. tropica* promastigotes by treatment with the selected callus extract (MTT assay).

|          | 1000 µg/mL | 500 µg/mL | 100 µg/mL | IC₅₀   |
|----------|------------|-----------|-----------|--------|
| Control  | 64.07 ± 3.1| 23.20 ± 1.5| 12.17 ± 0.8| 820    |
| BA       | 79.26 ± 3.9| 44.20 ± 2.5| 35.24 ± 2.1| 474.8  |
| 2,4-D    | 77.87 ± 3.5| 59.56 ± 2.8| 43.45 ± 2.5| 263.4  |
| BA + 2,4-D| 77.92 ± 3.6| 65.97 ± 3.1| 61.71 ± 3.0| 19.13  |

#### Apoptosis assay for estimation of anti-leishmanial potential in callus extracts

Apoptosis is a kind of heritably regulated cell death that controls the development of eukaryotic tissues by eliminating physiologically redundant, injured and odd somatic cells [39]. In order to elucidate the apoptosis generation ability of the callus cell lines against *L. tropica*, dual AO/EB staining method was employed.

Dual AO/EB fluorescent staining can detect basic morphological changes in apoptotic cells. Additionally, it also helps in identifying the normal cells from early apoptotic to late apoptotic and necrotic cells [39]. In this investigation, the callus extract derived from BA plus 2,4-D (1.5 mg/L, each) treated cultures, showed the best response at 1000 µg/mL. Where a number of early-stage apoptotic (EA) and late-stage apoptotic (LA) cells were observed at higher levels. Moreover, the callus extract established in response to BA (1.5 mg/L) induced EA cells, characterized by chromatin condensation at 1000 µg/mL (Figure 4). However, the control sample showed lesser apoptotic activity compared with the other callus cell lines tested at 1000 g/mL. EA cells were marked by granular yellow-green AO nuclear staining. LA cells were marked with concentrated and irregularly confined yellow nuclear EB staining. These visual characteristics of the *L. tropica* cells during apoptosis assay are due to the dead cells which increased up in volume and thus showed irregular yellowing and fluorescence [24]. We assume that AO when entered the normal and early apoptotic cells with intact membranes, caused green fluorescence on attachment to DNA. Further, EB can only pass through damaged membranes of cells, such as late apoptotic and dead cells, radiating orange-red fluorescence on attachment to concentrated DNA fragments or apoptotic bodies. Additionally, the staining of AO/EB has the ability to recognize slight DNA damages and thus can distinguish and measure the different morphological changes in the apoptotic cells [40].
Total phenolic, flavonoid content and free radical scavenger activity in the callus cultures

The callus cultures resulted in significant variations in the production of total phenolic and flavonoid content and DPPH free radical scavenger activity (Figure 5(a–c)). The callus cultures established in response to the combination of BA and 2,4-D (1.5 mg/L, each) produced higher levels of TPC (4.1 mg GAE/g). However, compared with BA higher level of TPC was detected in the callus grown at 2,4-D (1.5 mg/L). The lower value of TPC (2.0 mg GAE/g) was detected in the control sample (Figure 5(a)). In a similar study, the highest TPC (52 ± 0.56 mg) was detected in the callus cultures of Crataegus azarolus, established at 2.0 mg/L 2,4-D plus 1.0 mg/L BAP [30]. Unlike data on TPC, interestingly the flavonoid content (TFC) was observed at a higher level (2.8 mg GAE/g) in the callus grown at 2.0 mg/L 2,4-D. A combination of equal levels of BA plus 2,4-D considerable amount of flavonoids (1.7 mg QAE/g) were produced in callus cultures. The lowest flavonoid content (0.8 mg QAE/g) was detected in the control sample (Figure 5(b)). Generally, the biosynthesis of phenols and flavonoids occurs through the derivation of their carbon skeletons from two basic compounds, malonyl-CoA and p-coumaroyl-CoA in the phenylpropanoid pathway [41]. At the first step, the formation of the phenylpropanoid skeleton takes place in plants due to the deamination of L-phenylalanine and yields trans-cinnamic acid and ammonia with the help of phenylalanine ammonia lyase enzyme, which is considered as a key enzyme in the biosynthesis of these phenylpropanoids [42]. Plant cell produces a variety of defence metabolites in response to stress stimuli, such as phenolic acids, flavonoids, alkaloids, etc., having human health promoting attributes [43]. Phenylpropanoids are low molecular weight compounds having antioxidant properties which are useful against several disorders and have also shown ample activity against leishmaniasis [4].

In this study, the highest DPPH free radical scavenger activity (85%) was observed in the callus raised at BA plus 2,4-D (1.5 mg/L, each). It was followed by callus grown at 2,4-D (2.0 mg/L). The lowest antioxidant activity (43%) was observed in the control sample (Figure 5(c)). In another study, Ali and Abbasi, [32] reported maximum production of TPC (8.53 mg), TFC (7.8 mg) and highest antiradical activity (72.6%) in the callus tissues of Artemisia absinthium, established at 1.0 mg/L TDZ. The DPPH assay is based on the reaction of the free radicals with the scavengers, which combines hydrogen radicals from potential antioxidants. Mostly, the greater performance in scavenging DPPH free radicals in the callus tissues is due to the higher presence of secondary metabolites in the callus [44].

Determination of anti-leishmanial metabolites in callus extracts

For a comprehensive analysis of the anti-leishmanial potential of plant extracts, it is crucial to determine the putative metabolites in the plant sample, responsible for the enhanced anti-leishmanial activity. In this study, the selected plant samples used in the MTT anti-leishmanial activity were subjected to Gas chromatography-mass spectrometry (GC-MS) analysis. The GC-MS profiles revealed the presence of several metabolites at varying levels in the plant extracts (Figure 6). The callus extract from BA supplemented cultures showed production of 87% total metabolites and the control sample revealed biosynthesis of 83% total metabolites. Further, a significant increase in the total metabolites (98%) was observed in the callus raised at 2,4-D plus BA followed by callus extract grown at 2,4-D applied separately. In the list, the metabolites which were found at trace amount were ignored and those detected at different levels in all the selected plant extracts were taken for assessment of their comparative role in the anti-leishmanial activity. Significantly, higher levels of the metabolites, such as nerolidol (22%), pelletierine (18%), aspidin (15%) and ascaridole (11%) were detected in the callus extract grown at BA plus 2,4-D (1.5 mg/L, each) (Figure 6). The other metabolites, such as 3-carene and arecoline were found abundantly in the 2,4-D treated callus. Interestingly in the control sample, β-pinene and valencene were detected at higher quantities. As the data on the anti-leishmanial activity (Figure 3) of the selected extracts showed, higher activity (IC50:19.13 μg/mL) for the callus extract established under the effect of BA plus 2,4-D (1.5 mg/L, each), the same sample showed higher level of nerolidol (Figure 6). The elevated level of nerolidol might be correlated to the apoptosis of cells, leading to the death of L.
tropica promastigotes. Further, nerolidol has reportedly shown very fine anti-leishmanial activity against several leishmanial species [45,46]. Nerolidol can cause morphological changes in the treated parasite including cellular and chromatin material shrinkage, exposure of phosphatidylserine (an indicator of the early stage of apoptosis) and DNA damage (an indicator of the last stage of apoptosis) [46]. Nonetheless, aspidin and ascaridole, detected at considerable quantities in this study are the potent natural products, used in the treatment of worms. They play an active role against intestinal cestodes by paralyzing the worm’s muscles [47].

Conclusions
Leishmaniasis is one of the alarming infectious diseases with many adversaries on human health. The issues of efficacy and side effects in the available medicines demand other alternatives in the treatment of leishmaniasis. Artemisia scoparia is an important medicinal herb and can be exploited for the production of important anti-leishmanial metabolites. In this study, for the first time, callus cultures of A. scoparia were established and were used for the anti-leishmanial activities using MTT and cell apoptosis assays. The PGRs, such as 2,4-D in combo with BA at optimal levels were found inductive for the biosynthesis of putatively known anti-leishmanial natural products. This protocol can be scaled up for commercial production of the important metabolites; those can be used in the preparation of effective phytomedicines in the treatment of leishmaniasis.

Disclosure statement
The authors declare that they have no conflict of interest regarding this publication.
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