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Preliminary Evaluation of a Four-Spice-Based Biphasic Culture Medium for In Vitro Growth of *Leishmania donovani*-Complex Promastigotes

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Abstract

Background: Affordable culture formulations may be useful for laboratory propagation of Leishmania promastigotes where conventional media are costly or difficult to obtain. **Objective**: To evaluate whether a four-spice formulation could support promastigote growth when used as the solid phase, the liquid phase, or both phases of a biphasic culture system, in comparison with modified Novy–MacNeal–Nicolle (NNN) medium. **Methods**: Two plant lots were used and three experiments were performed with 3 clinical isolates that were reported as members of the L. donovani complex. The basic design was based on 64 culture tubes, with 4 tubes per group. G1 – was the solid phase of NNN with plant liquid; G2 – was the solid phase of plant with oral rehydration solution (ORS); and G3 (control) – was the solid phase of NNN with oral rehydration solution (ORS). G4 was an agar–ORS control without a plant. In experiment 1, there were also additional groups that consisted of a fully plant-based formulation (G5), agar with plant liquid (G6), NNN with a plant liquid that was pH and osmolality adjusted (G7), and an unwashed-inoculum carry-over control (G8). Tube-level area under the growth curve (AUC) days 1-20 was the primary outcome. Secondary tests were repeated measures growth analysis, viability, culture pH, medium composition and serial passage, macrophage infectivity, and direct ingredient cost. **Results**: G4 and G8 had little growth with AUC of 1.4% and 2.7% of G3, respectively. In all three experiments, G1 and G2 had an AUC of 77.4–83.8% and 85.9–86.9% of G3, respectively, and was significantly less than G3 ($P < 0.001$ for each comparison). In experiment 1, AUC values were 67.9% for G5, 26.4% for G6, and 91.8% for G7. Day-20 viability was 77.3% in G3, compared with 55.3% in G1, 57.8% in G2, and 46.5% in G5. Passage retention for G1 and G2 ranged from 83.1 to 87.7% while for G3 it was 95.3 to 99.3%. The results of the pooled macrophage-infection were lower in G1 and G2 than in G3. The cost of ingredients per tube was 37.8% of the control for G2 and 42.4% of the control for G5. **Conclusions**: In vitro growth of promastigotes reported as L. donovani-complex isolates was supported by the tested spice-based phases, with growth and a number of late-culture parameters still being lower when compared to the NNN-based control. G2 (plant solid phase with ORS) had the best growth/ingredient cost ratio of the plant-based formulations tested. These preliminary findings do not establish equivalence to NNN and do not support use for clinical diagnosis, parasite isolation, or drug-susceptibility testing.

Keywords: *Leishmania donovani* complex; promastigotes; culture medium; NNN medium.

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1. Introduction

Visceral leishmaniasis is a serious illness primarily caused by Leishmania donovani and L. infantum and transmitted by phlebotomine sandflies. Long-lasting fever, hepatosplenomegaly, and weight loss are clinical symptoms [1–4]. There are only a few drugs available for treatment, including miltefosine, amphotericin B, pentavalent antimonials, and pentamidine, and toxicity and cost are significant issues, as is treatment failure [5,6]. The disease remains endemic in Africa, Asia and the Middle East [7,8].

Studies on differentiation processes, parasite pathogenesis, and screening of potential drugs and molecular targets are conducted using in vitro-grown promastigotes [9–11]. The culture system should ensure the reproducible growth of parasites and be accessible and economical, especially in low-resource laboratories.

Leishmania can be cultured in semi-solid, biphasic or liquid culture systems. One of the most popular biphasic formulations is Novy–MacNeal–Nicolle (NNN) medium, which features a solid phase with blood and a fluid overlay. While it is relatively simple to prepare and has been developed, it may be affected by differences in blood sources and batches [12]. Many liquid media, like RPMI-1640, M199, and liver infusion tryptose, require serum components that add to the expense and reduce the availability of the media [13–15].

Several alternative formulations were investigated, including media using human urine, bee pollen, lentil preparations, and other inexpensive ingredients, as well as liquid systems that do not contain serum or blood [15–23]. These studies provide a rationale for evaluating locally available materials as culture components. However, they do not conclude that any specific plant material can provide the nutritional and physicochemical benefits of conventional media.

Leishmania promastigotes have established nutritional requirements, but sometimes the role that is played by different nutrients in the complex media is not clearly understood. In addition to purine salvage [24], the parasites need iron [25] and folate [26], as well as amino acids, carbohydrates, vitamins, and inorganic salts [9]. These components are provided by the nutrient base, dextrose and blood in NNN-based formulations.

Different varieties of spices contain different levels of carbohydrates, proteins, minerals, vitamins and phytochemicals, including cardamom (Elettaria cardamomum), clove (Syzygium aromaticum), star anise (Illicium verum) and black seed (Nigella sativa) [27–31]. Some constituents have shown antimicrobial activity, and extracts of N. sativa have exhibited antileishmanial activity in assays with other Leishmania species [32,33]. These properties offer competing alternatives: a spice preparation could supply nutrients or inhibit growth of parasites, or both, depending on the nutrient content, amount, and preparation of the spice. It is therefore important to evaluate its net effect on promastigotes of L. donovani-complex experimentally.

In the present study, a fixed four spice mixture was investigated as part of biphasic culture formulations. The main comparisons included were the replacement of the liquid phase (G1) or solid phase (G2) in the NNN based system, while G3 was used as the NNN-solid-plus-ORS control. An

agar control (G4) and unwashed-inoculum control (G8, experiment 1) were also grown to determine if observed growth is due to carry-over or non-plant factors. Other experiment-1 groups tested a plant-based formulation (G5), plant liquid without NNN solid (G6), and plant liquid pH and osmolality adjusted (G7). Growth, viability, passage retention, macrophage infectivity, medium properties and direct ingredient costs were measured. The study was not intended as a validation of an alternative medium for diagnosis, clinical isolation or drug susceptibility testing.

2. Materials and methods

2.1 Parasites and maintenance

Three clinical isolates were obtained from the College of Science, University of Baghdad. For experiment 1, isolate LD-UoB-01 was used; for experiment 2, isolate LD-UoB-02; and for experiment 3, isolate LD-UoB-03, which were reported to have been collected from different patients. The promastigotes were cultured in a biphasic NNN medium [34] at 27 °C with 8-day transfers. The LITSR/L5.8S primers were used to perform ITS1-PCR, generating a 320 bp amplicon. HaeIII digestion resulted in a pattern of about 200 and 140 bp fragments, typical of the L. donovani complex. No sequencing or strain typing was done. For this reason, the molecular results are useful only for identification at the complex level; species-level identification was not performed as part of this study, and species-level identification is based on information provided by the source laboratory.

2.2 Plant materials

Cardamom, clove, star anise and black seed were bought from local markets in Baghdad. Lot A, used in experiment 2, and lot B, used in experiments 1 and 3, were obtained from two different suppliers. Botanical identity was determined from suppliers' labels alone; voucher specimens and independent botanical authentication were not available. All dried materials were kept in closed glass jars in the dark at ~25 °C for less than three weeks prior to use. Cardamom, clove buds, whole of star anise and the whole black seeds were not washed. For each preparation 6 g of spices (1.5 g of each spice) were placed in 100 mL of distilled water and blended for 2 min. No extraction or filtration was done and remained insoluble particles. One mixture and one concentration was tested. Proximate composition, iron, and folate were also determined in lot A (Supplementary Data) and total phenolics and marker compounds were measured for both lots.

2.3 Preparation of culture phases

The compositions of the solid and liquid phases are summarised in Table 1. The NNN solid phase was a modified blood-agar–dextrose formulation. The four-spice mixture mentioned above was added to plant solid and plant liquid preparations. Solid-phase pH was adjusted with 1 N NaOH before autoclaving at 121 °C for 20 min. Plant liquid was autoclaved (121 °C, 15 min) without pH adjustment (except for G7). One WHO reduced-osmolarity sachet was dissolved in 1 L of distilled water and autoclaved at 121 °C for 15 min to prepare ORS. For G7, the plant liquid (initial pH 5.6) was then adjusted to pH 7.4 using 1N NaOH (0.6 mL/100 mL) added dropwise, and NaCl (0.4336 g/100 mL) was added to raise the measured osmolality from 109 mOsm/kg to the target osmolality of 247 mOsm/kg in the ORS range. After autoclaving, the measured pH and osmolality were 7.3 and 251 mOsm/kg, respectively. Due to the correlated change of both pH and osmolality, it is impossible to separate their different effects.

Gentamicin sulfate was added to the phases after cooling: 0.25 mL of a stock solution of 40 mg/mL gentamicin per 120 mL of NNN solid and 100 mL of ORS aliquots, and 2.5 mL of a working solution of 4 mg/mL gentamicin per 100 mL of plant solid or plant liquid, respectively. One reduced-iron pellet (approximately 1 mg elemental iron) was added to each solid-phase tube. As a sterility test, solid-phase tubes were incubated for 24 h at 37 °C, and there was no sign of growth. The amount of solid phase added to each tube was 4 mL, the liquid phase was 4.0 mL, and 0.2 mL of the inoculum was added, with a final liquid volume of about 4.2 mL. Both the experimental and stock cultures were incubated at 27 °C.

Table 1. Composition of the culture phases (solid phases and plant liquid per 100 mL; ORS per litre).

Component	NNN solid	Plant solid	Agar solid	Plant liquid	ORS liquid
BHI / dextrose	3.7 g / 1 g	–	–	–	–
Agar	2 g	2 g	2 g	–	–
Blood	20 mL	–	–	–	–
Cardamom, clove, black seed, star anise	–	1.5 g each	–	1.5 g each	–
NaCl / KCl / trisodium citrate/glucose	–	–	–	–	2.6 / 1.5 / 2.9 / 13.5 g/L
Gentamicin (final)	~83 µg/mL	100 µg/mL	100 µg/mL	100 µg/mL	100 µg/mL (0.25 mL of 40 mg/mL per 100 mL)
Iron pellet (~1 mg)	1 per tube	1 per tube	1 per tube	–	–
Distilled water	100 mL	100 mL	100 mL	100 mL	1 L
pH adjustment	1 N NaOH to 7.4	1 N NaOH to 7.4	1 N NaOH to 7.4	none	none
Autoclaving (121 °C)	20 min	20 min	20 min	15 min	15 min
Used in groups	G1, G3, G7	G2, G5	G4, G6, G8	G1, G5, G6 (adjusted for G7)	G2, G3, G4, G8

The final concentration of gentamicin was around 83 µg/mL in NNN solid and 100 µg/mL in the other forms. The iron pellet represented a nominal addition, and dissolved iron, measured separately (Table 7), should not be interpreted as the total iron content of the pellet or solid phase.

2.4 Experimental design

Eight groups were created (Table 2). The primary data was taken from 64 tubes with 4 tubes per group (32 from experiment 1 and 16 from each of experiment 2 and experiment 3). Also, 48 satellite tubes for pH evaluation and 128 passage tubes were added to the contamination log bringing the total to 176 more tubes. G4 was the plant-free negative control. In all three experiments, the inocula were washed twice with phosphate-buffered saline (PBS; 10mL, 3,000 ×g, 10min) and then resuspended in saline at twice the stock inocula volume. Washing resulted in 94- 95% recovery of cells, with an estimated residual inoculum of 0.024% v/v, while the unwashed inoculum had an estimated residual inoculum of 2.4% v/v. G1–G8 were included in Experiment 1, and an unwashed-inoculum carry-over control was included as G8. In experiments 2 and 3 G1–G4 were used along with two other isolates. In this instance, the two plant lots were not fully crossed with the three isolates, with only one lot (plant lot A) being tested with isolate 02 and only the other lot (plant lot B) being tested with isolate 01 and isolate 03, so that no separation of isolate effect and plant-lot effect is possible and no isolate-by-lot interaction is estimated.

Table 2. Experimental groups and culture-phase combinations.

Group	Solid phase	Liquid phase	Purpose	Experiments
G1	NNN	Plant liquid	Plant liquid substituted for ORS	1–3
G2	Plant solid	ORS	Plant solid substituted for BHI-dextrose-blood	1–3
G3	NNN	ORS	Control (NNN medium)	1–3
G4	Agar	ORS	Plant-free negative control (carry-over, agar, iron, gentamicin, ORS)	1–3
G5	Plant solid	Plant liquid	Fully plant-based medium	1
G6	Agar	Plant liquid	Plant liquid without NNN or plant solid	1
G7	NNN	Plant liquid, pH 7.3 and 251 mOsm/kg measured (target 247)	Combined pH/osmolality adjustment of plant liquid over NNN solid	1
G8	Agar	ORS	Unwashed inoculum (carry-over control)	1

Table 3. Design of the three experiments (four tubes per group).

Exp	Isolate	Plant lot	Groups	Inoculum	Counts	Day 0
1	LD-UoB-01	B	G1–G8	Washed (22,619/mL)	Second observer, blinded	04 May 2025
2	LD-UoB-02	A	G1–G4	Washed (20,238/mL)	Second observer, blinded	18 May 2025
3	LD-UoB-03	B	G1–G4	Washed (23,810/mL)	Second observer, blinded	24 May 2025

Inoculum concentrations are the final concentrations in each tube, calculated from the 0.2 mL inoculum volume in a final liquid volume of about 4.2 mL. Tubes were coded prior to counting to aid blinded assessment.

2.5 Counting, sampling, and observation

Promastigotes were counted on days 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 using an Improved Neubauer haemocytometer (0.1-mm depth). The number of squares counted depended on the presumption of the cell density and dilution. The concentration was determined by dividing the cells counted by the number of squares counted, then multiplying by the dilution factor and 10^4 cells/mL. When necessary, samples were diluted in saline-formalin: undiluted samples were used on days 1 and 20; 1:2 was necessary on days 2, 16, and 18; and 1:4 was necessary on days 4, 6, 8, 10, 12, and 14. For day 20 (low density) 16 large squares were counted in each chamber. Counts for each experiment were made by a second observer who was unaware of the group allocation. No repeat chamber loading was done. The liquid layer was stirred gently without affecting the solid phase, prior to taking samples. In total, 11 counts were taken, taking out 20 µL every count (or 0.22 mL, 5.2% of a total volume of 4.2 mL liquid). About 15% of the liquid was removed using trypan blue sampling prior to day 20 in experiment 1 (0.2 mL removed at days 8 and 14, with the remaining liquid being sampled at day 20). Motility and morphology were qualitatively assessed at each count. Level of contamination was assessed by microscopy and turbidity/colour changes. All culture tubes were tested. Liquid-phase bottles were incubated for 48 h at 37 °C, and a 48-hour blood agar culture was made with 10 µL of culture liquid obtained from a terminal streak of culture at 37 °C.

To determine the level of counting agreement (Section 3.7), 12 stored samples were recounted independently by two observers from experiment 1. The parasite-sized particles were counted in the plant liquid free of cells and the morphology and size of these particles compared with promastigotes

in order to investigate particle interference. The known spike recovery was also measured in ORS, plant liquid and distilled water.

2.6 Viability, culture pH, medium characterisation, serial passage, and infectivity

On days 8 and 14, 0.2-mL aliquots of the samples were stained with trypan blue to estimate viability, and the remaining liquid layer was stained to determine viability on day 20. Samples were centrifuged at 3,000 × g for 5 min and then resuspended in a 1:1 ratio with trypan blue (0.4%). The first 200 cells were scored after centrifugation. Viability was not determined when no cells were detected (including G4 and G8 at the corresponding time points).

In experiment 1, the pH of the culture was determined with satellite tubes using one tube per group per day. These tubes were incubated with the counted tubes but were not used to count the parasites. On day 7, measurements were taken for 48 satellite tubes. pH (at 25 °C) and osmolality from a freezing-point osmometer were also measured for retained aliquots from each preparation batch after autoclaving. The melting of solid phases was performed at 45 °C, and the plant liquid was clarified and was centrifuged at 3000 × g for 5 min prior to measurement.

For final-medium characterisation, filtered autoclaved phases (0.22 µm) were analysed in triplicate, to remove any insoluble material. Soluble protein, reducing sugar, dissolved iron, ferrous iron (ferrozine method), eugenol and trans-anethole were measured. The level of total phenolics and folate content in the plant liquid was determined before and after autoclaving. The reported in vitro IC_{50} of eugenol against promastigotes of L. donovani was used for reference [35]. The proximate composition, iron and folate contents of dry plant materials also were analysed (Supplementary Data).

The infectivity by serial passage and by phagocytes was determined as follows. On day 8 of each passage, 0.2 mL of each day-8 culture was removed and placed in a new culture tube, four lineages per group, and counted on day 8 of each passage (P1–P3). G1, G2, G3 and G5 were passaged in experiment 1; in experiments 2 and 3, an attempt was made to passage G1 – G4. In experiments 2 and 3, all of the G4 lineages were eradicated by P1. The passage retention was the number of parasites counted at P3 on Day 8 divided by the number of parasites counted at P1 on Day 8 and expressed as a percentage. The human monocytic cells, THP-1 [36] were cultured in RPMI-1640, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and penicillin/streptomycin. 5×10^4 cells per well (300 µL) were added to an 8-well chamber slide. Differentiation was achieved by the addition of phorbol 12-myristate 13-acetate (PMA; 25 ng/mL) for 48 h, then returned to normal culture medium without PMA for 24 h. No antibiotics were used in differentiation and infection.

Before infection, more than 90% of THP-1 cells were seen to be adherent and spread (phase-contrast microscopy). The THP-1 passage numbers at infection were 9, 11, and 13 in experiments 1, 2, and 3, respectively. The liquid phase of day-8 culture was harvested, and parasites were washed in PBS (3000 × g, for 10 min/wash) and then resuspended in antibiotic-free RPMI-1640 with 10% FBS at 2×10^6 parasites/mL. The source tube varied from experiment to experiment: In experiment 1 the source tube was the day-8 satellite tube after the pH measurement, and in experiments 2 and 3 the source tube was the lineage-4 P1 tube after the P2 inoculum was removed. No metacyclic enrichment or other parasite selection was performed; the entire day-8 population was used.

Three wells were set up for each group and isolate, each containing 250 µL of 5×10^5 parasites; two wells were set up as uninfected controls for each run. Parasites and macrophages were cultured at 37 °C and 5% CO_2 for 4 hours. The non-adherent parasites were then washed three times with warm

PBS, before the addition of fresh, antibiotic-free media. The incubation was continued for an additional 20 h (total 24 h since the onset of the infection). Air drying was performed and then staining with 10% Giemsa in pH 7.2 buffer for 20 min and air drying again was done. The first 100 macrophages in every well in at least 5 oil-immersion fields (100×) were scored by a blinded observer. The infection percentage was the percentage of macrophages with at least one amastigote while the amastigote burden was the number of amastigotes in 100 macrophages.

In the uninfected controls no macrophages were infected. All three isolates were evaluated with respect to their G1, G2 and G3, whereas evaluate only for isolate 01 with respect to G5. The testing of each isolate was performed on a different day, in a different run, and with a separate passage of THP-1. All ingredient costs were determined by the local price of the ingredients in the culture and the volume (mL) of ingredients required to make one batch/tube (4 mL solids + 4 mL liquid). The cost estimates are provided for each of the eight formulations, including agar solid and NaCl and NaOH additions to adjusted plant liquid. These are not included: Labour, plasticware, electricity, quality assurance, storage, waste disposal, and PBS used for washing.

2.7 Statistical analysis

The main outcome was tube-level AUC, which was obtained by trapezoidal integration of the parasite counts on days 1 to 20 and was reported as 10^4 parasite-days/mL. This summary measure was examined with one-way ANOVA treatment comparisons within each experiment and followed by Tukey HSD post hoc comparisons (4 tubes per treatment). Secondary growth analysis involved using repeated measurements with tube as the repeated unit, day as the within-tube factor, and group as the between-tube factor. The counts were log transformed ($\log_{10}(\text{count} + 0.5)$). Tube, day and group-by-day effects were tested and Greenhouse–Geisser corrections were used for non-sphericity ($\varepsilon = 0.336$–0.525), and corrected P values are reported in Supplementary Table S1. Quasi-binomial generalised linear models with estimated dispersion were used to analyse viability proportions.

Four lineages per group were used for passage retention. The infectivity outcomes referred to three wells per group per isolate; the 100 macrophages in each well were used as subsamples and were not independent experimental replications. For passage and infectivity results, one-way ANOVA plus Tukey comparisons were employed within experiments. The comparisons between G1-G3 were made using a pooled two-way ANOVA (group and isolate). Primary AUC analysis did not pool the data across experiments. The plant lot was confounded in the experiment and isolate and only one run per isolate was done for passage and infectivity. AUC ratios were therefore compared descriptively between experiments to evaluate consistency to G3.

Pooled variance estimates were used to conduct pairwise comparisons between the growing groups. In experiment 1, with four tubes per group, the normality and variance-homogeneity could not be reliably determined, and the near zero groups (G4 and G8) exhibited variance behaviour that differed from the growing groups. P values are measures of variation between tubes within the isolate and experimental run and should not be given as estimates of between-isolate biological variability. Peak counts and percentages vs. G3 are descriptive. A non-inferiority or equivalence analysis was not planned, so no statistical differences or similarities can be used to demonstrate equivalence to NNN.

3. Results

3.1 Growth kinetics across the three experiments

In general, according to all three experiments, the highest number of parasites was recorded on day 8, and by day 20 (without subculture) it decreased (Figure 1). The highest AUC was obtained in each experiment by G3 (Table 4). G1 reached 77.4–83.8% of the G3 AUC, whereas G2 reached 85.9–86.9%. Day-8 peak counts were 78.5–87.9% of the control for G1 and 88.7–90.3% for G2. In all experiments AUC for both G1 and G2 were lower than G3 (Tukey $P < 0.001$ for both contrasts). Experiment 1 ($P < 0.001$) and experiment 2 ($P = 0.005$) had a higher AUC for G2 compared with G1, but this was not statistically significant in experiment 3 ($P = 0.093$). This overall ranking was also found in the three isolates and two plant lots. It should be noted, however, that isolate and lot were not completely crossed, which means that differences between isolate and lot cannot be separated. There were also group and group-by-day effects observed in each experiment (group effect, $P < 0.001$; Greenhouse–Geisser-adjusted group-by-day effect, $P < 0.001$), which suggested that differences in the overall counts, and in the shape of the growth curves, existed between the groups, especially between days 6 and 14.

Table 4. Tube-level AUC ($\times 10^4$ parasite-days/mL; mean ± SD, n = 4 tubes per group) and day-8 peak as a percentage of G3, by experiment.

Exp	G1	G2	G3 (control)	G4	ANOVA F; P	Peak, % of G3
Exp 1	670.8 ± 8.4 (77.4%)	753.0 ± 16.9 (86.9%)	866.8 ± 13.6 (100.0%)	12.4 ± 0.2 (1.4%)	4965.1; <0.001	G1 78.5; G2 90.3
Exp 2	656.7 ± 7.4 (83.3%)	679.0 ± 8.1 (86.2%)	788.1 ± 9.5 (100.0%)	10.4 ± 1.7 (1.3%)	9367.5; <0.001	G1 87.9; G2 90.2
Exp 3	752.6 ± 2.4 (83.8%)	771.5 ± 15.6 (85.9%)	898.1 ± 13.0 (100.0%)	11.8 ± 0.9 (1.3%)	6197.6; <0.001	G1 82.0; G2 88.7

AUC percentages are calculated relative to G3 within the same experiment. The ANOVA included all groups tested in that experiment (eight groups in experiment 1 and four groups in experiments 2 and 3). All G1-versus-G3 and G2-versus-G3 Tukey contrasts had $P < 0.001$.

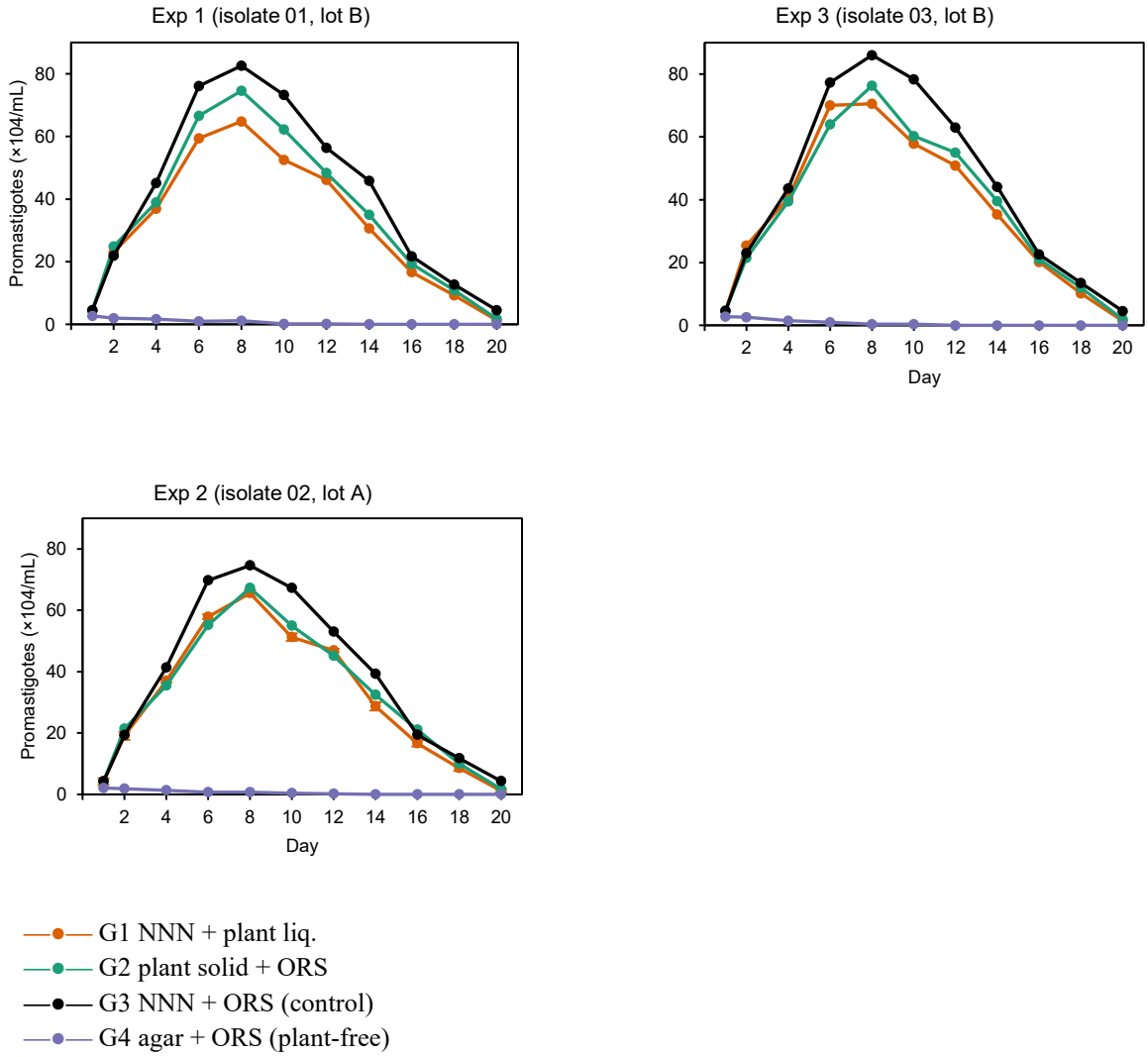

Figure 1. Growth curves (mean ± SD, n = 4 tubes per group) in the three experiments; G4 (plant-free control) is shown in each.

3.2 Negative and inoculum carry-over controls

In all experiments, G4 (plant-free agar–ORS) did not support parasite growth. Population counts on day 1 were about 1.0–1.2 times the inoculum level and then decreased to 0 or close to 0. In experiments 1, 2 and 3, the AUC was 12.4 × 10^4, 10.4 × 10^4 and 11.8 × 10^4 parasite-days/mL, respectively, which amounts to around 1.3–1.4% of G3. In experiment 1, unwashed inoculum (spent NNN liquid 2.4% v/v) was used and the growth in G8 was also minimal (AUC 23.5, 2.7% of G3). G8 was not significantly different from G4 (Tukey P = 0.76; mean difference −11.1, 95% CI −34.4 to 12.2). These controls demonstrate that the modest increase in growth for G4 and G8 growth was not enough to account for the significantly increased growth seen with the plant containing formulations. They are not however, isolating the contribution of all the individual medium component.

3.3 Comparison of the eight formulations in experiment 1

The eight groups in experiment 1 had significantly different AUC values (F = 4965, P < 0.001; Table 5 and Figure 2). Relative to G3, AUC was 91.8% in G7, 86.9% in G2, 77.4% in G1, 67.9% in G5,

and 26.4% in G6; G4 and G8 remained below 3% of the control. Pairwise comparisons among G1, G2, G3, G5, G6, and G7 were statistically significant (Tukey $P < 0.001$; Supplementary Table S2). The addition of pH and osmolality adjustment to G7 increased AUC by 124.5 units compared with G1, which is about 63.5% of the AUC difference between G1 and G3. However, G7 were still 71.4 units lower than G3. The mean AUC value of G5 (plant solid plus plant liquid) was 164.4 lower than G2 (plant solid plus ORS). G6 (agar plus plant liquid) showed a day-8 peak of 24.75 × 10^4/mL and an AUC 216.4 units higher than G4. G7 was performed at the same time as pH and osmolality were adjusted, so the improvement cannot be attributed to one or the other of these factors alone.

Table 5. Experiment 1: AUC and peak parasite count by group (mean ± SD, n = 4 tubes).

Group	Composition	AUC	AUC, % of G3	Peak (×10^4/mL), day	Peak, % of G3
G1	NNN + plant liquid	670.8 ± 8.4	77.4	64.81, day 8	78.5
G2	Plant solid + ORS	753.0 ± 16.9	86.9	74.56, day 8	90.3
G3	NNN + ORS (control)	866.8 ± 13.6	100.0	82.56, day 8	100.0
G4	Agar + ORS	12.4 ± 0.2	1.4	2.69, day 1	3.3
G5	Plant solid + plant liquid	588.7 ± 9.1	67.9	58.00, day 8	70.2
G6	Agar + plant liquid	228.8 ± 4.6	26.4	24.75, day 8	30.0
G7	NNN + adjusted plant liquid	795.3 ± 11.7	91.8	73.69, day 8	89.3
G8	Agar + ORS, unwashed inoculum	23.5 ± 2.8	2.7	3.06, day 2	3.7

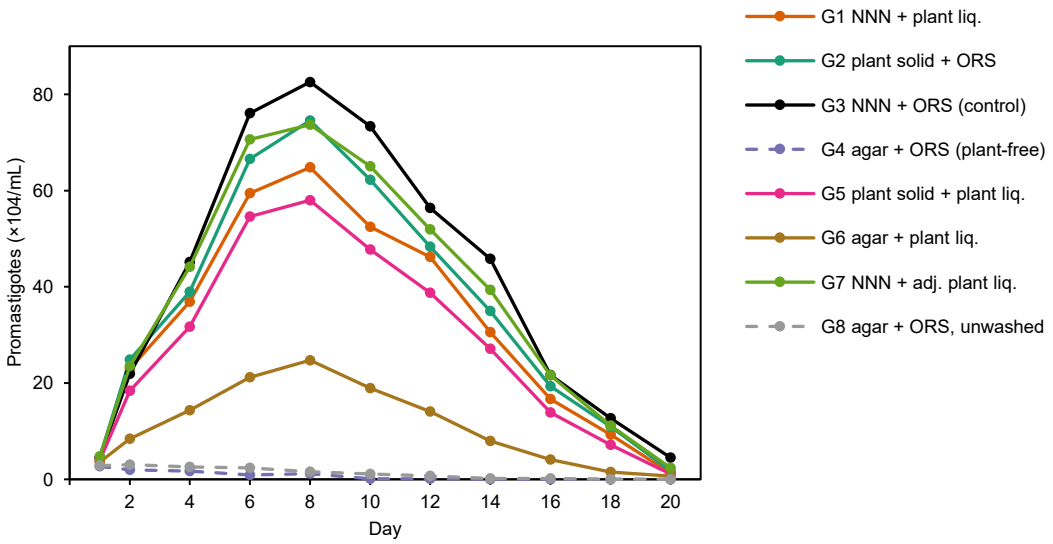

Figure 2. Experiment 1 growth curves for all eight groups (mean ± SD, n = 4 tubes per group). Dashed lines indicate the negative (G4) and inoculum carry-over (G8) controls.

3.4 Viability

In experiment 1, trypan blue viability on day 8 was 95.0–97.9% in G1, G2, G3, G5, and G7, 89.0% in G6, and approximately 69% in G4 and G8, where few cells remained (Table 6). On day 14, the highest values were observed in G3 (91.6%) and G7 (91.1%). By day 20, viability was 77.3% in G3, 67.3% in G7, 57.8% in G2, 55.3% in G1, 46.5% in G5, and 32.6% in G6. Group effects were reported for each day (day 8: $F = 29.41$, $df = 7,24$; day 14: $F = 30.09$, $df = 6,21$; day 20: $F = 29.96$, $df = 5,18$; all $P < 0.001$). The viability at day 20 was higher in G3 than in G1 and G2. The results should be

viewed in the context of a decreasing batch culture as these late measurements were made at parasite counts of ~1–5 × 10^4/mL in the corresponding cultures.

Table 6. Experiment 1: trypan-blue viability (%, mean of four tubes; 200 cells scored per tube).

Group	Day 8	Day 14	Day 20
G1	95.1	87.0	55.3
G2	95.5	86.8	57.8
G3	96.6	91.6	77.3
G4	68.9	no cells	no cells
G5	95.0	84.4	46.5
G6	89.0	66.4	32.6
G7	97.9	91.1	67.3
G8	68.6	48.3	no cells

Quasi-binomial GLM group effects were $P < 0.001$ on each day. Groups with no detectable cells (G4 on days 14 and 20; G8 on day 20) were excluded from the corresponding models.

3.5 Culture pH and physicochemical characteristics

Measured pH after autoclaving was 7.3, 7.2, 5.6 and 7.3 for NNN solid, plant solid, plant liquid and ORS respectively. The osmolality values were 336, 158, 109 and 247 mOsm/kg respectively. By day 20, pH in plant-liquid groups had increased to 6.8 in G1, 6.6 in G5, and 6.0 in G6. The pH change in the uninoculated tubes (6.7, 6.5, and 5.9, respectively) also was similar, thus concluding that most of the pH change was not due to parasite growth. The pH of ORS was kept in the range of about 7.2-7.7. These measurements were descriptive and made on a single satellite tube per group per day (Supplementary Table S3).

3.6 Chemical Characterisation of the finished media

Soluble protein concentration in filtered autoclaved plant solid and plant liquid was 1.7 mg/mL and 1.9 mg/mL, respectively, while reducing-sugar concentration in the plant solid and plant liquid were 4.1 and 3.9 mg/mL, respectively (Table 7). The values obtained for NNN solid were 4.8 mg/mL protein and 12.1 mg/mL reducing sugar. The iron pellet was a nominal addition of approximately 250 µg/mL, of which only a small fraction was identified as dissolved iron in the plant solid. The dissolved-iron assay indicates what amount of the added iron is soluble and should be considered as such, and not as the total amount of iron added in the pellet.

Eugenol concentrations were 37.2 µg/mL in plant solid and 41.6 µg/mL in plant liquid; trans-anethole concentrations were 11.4 and 12.3 µg/mL, respectively. The measured eugenol concentration in plant liquid was approximately 40% of the reported L. donovani-promastigote IC_{50} of 105 µg/mL [35]. No corresponding IC_{50} reference was available for trans-anethole, and other constituents or combinations were not evaluated. Folate retention in plant liquid after autoclaving was 55.4% (0.92 to 0.51 µg/100 mL), while phenolic retention was 85.7%; 48.6% of the phenolics recoverable by complete extraction were present in the liquid fraction.

Table 7. Characteristics of finished autoclaved phases (0.22-µm filtrate; mean of three determinations).

Phase	Soluble protein (mg/mL)	Reducing sugar (mg/mL)	Dissolved iron (µg/mL)	Fe^{2+} (µg/mL)	Eugenol (µg/mL)	trans-Anethole (µg/mL)
NNN solid (melted)	4.8	12.1	62.4	1.9	0	0
Plant solid (melted)	1.7	4.1	4.8	3.1	37.2	11.4
Agar solid (melted)	0	0.3	0.6	0.2	0	0
Plant liquid	1.9	3.9	2.6	1.9	41.6	12.3
ORS liquid	0	13.5	0.2	0.1	0	0

3.7 Counting assessment and comparison of plant lots

The cell-free plant liquid had an approximate number of parasite-sized particles of 2.9×10^4/mL. The particles were differentiated from promastigotes in their morphology and size when being counted. The recovery of a known parasite spike was 99.7% in ORS, 95.9% in plant liquid and 82.2% in distilled water. The absolute difference and the mean bias for 12 stored samples recounted by two observers were 1.7% and +0.35%, respectively. There were only slight differences between the 2 lot plant total phenolics per ingredient and between the measured plant marker compounds (eugenol, trans-anethole, α-terpinyl acetate and thymoquinone) of not more than 8.2% and 3 relative-area points, respectively. The difference between the lot A (experiment 2) and the average of lot B (experiments 1 and 3) was less than 3 percentage points for both G1/G3 and G2/G3 AUC ratios. Observations are descriptive, and the isolate by lot design is incomplete so that the lot and isolate effects cannot be independently estimated.

3.8 Serial passage and macrophage infectivity

For each isolate, however, the passage retention (day-8 count at P3/at P1) was less in G1 and G2 than G3. G3 retention ranged from 95.3% to 99.3%, compared with 83.1–84.4% for G1 and 85.4–87.7% for G2. All three isolates had within-isolate differences (all $P < 0.01$) but not significantly different between each G1 and G2 (all $P \geq 0.15$). Pooled G1-G3 analysis revealed a group effect ($P < 0.001$), and no isolate effect ($P = 0.13$) or group-by-isolate interaction ($P = 0.80$). G5 retention was 78.1% in isolate 01 only; it was lower than G3 ($P < 0.001$) and was not clearly different from G1 ($P = 0.28$) or G2 ($P = 0.064$). In both experiments 2 and 3, all G4 lineages were wiped out by P1.

The level of infection of the parasites was numerically lower in the presence of plants in the formulations as compared to G3. In isolate 01, there were differences in the percentage of infected macrophages between the groups ($P = 0.003$), with G5 having significantly fewer infected macrophages than G3 ($P = 0.002$) and G1 ($P = 0.033$) while group G3 had no significant difference from groups G1 or G2 in this measure. Amastigote burden differed among groups ($P < 0.001$), with G3 (225.3 per 100 macrophages) exceeding G1 (178.7), G2 (182.7), and G5 (140.7). Pooled analyses of G1–G3 (isolates 01–03) reported group effects on both percent infected and amastigote burden ($P < 0.001$). G1 and G2 were not significantly different, but were both lower than G3. Isolate effects were also reported, and there was no group by isolate interaction detected. G5 was evaluated in only one isolate and should not be compared between isolates. Infection was measured at 24 h, but

intracellular survival was not analysed beyond this time and proportions of metacyclics were not analysed.

Table 8. Serial-passage retention (P3 as a percentage of P1; mean of four lineages) and macrophage infectivity (mean of three wells) by isolate.

Isolate (exp)	Group	Passage retention, %	% infected	Amastigotes /100 macrophages
01 (1)	G1	83.1	58.3	178.7
01 (1)	G2	85.4	57.0	182.7
01 (1)	G3	95.3	62.7	225.3
01 (1)	G5	78.1	51.3	140.7
02 (2)	G1	84.4	55.0	169.3
02 (2)	G2	86.2	54.7	159.3
02 (2)	G3	96.5	60.3	191.0
03 (3)	G1	84.0	58.7	180.7
03 (3)	G2	87.7	59.0	184.0
03 (3)	G3	99.3	67.0	221.3

For isolate 01, day-8 counts at P1 were 67.8 (G1), 70.2 (G2), 81.1 (G3), and 57.9 (G5) × 10^4/mL; corresponding P3 counts were 56.3, 59.9, 77.3, and 45.2 × 10^4/mL. G4 lineages in isolates 02 and 03 were lost by P1 and are not presented.

3.9 Morphology and contamination monitoring

Only the groups which were able to grow (G1–G3 and G5–G7) were found to contain motile promastigotes of the characteristic elongated shape. There were no tubes reported as being contaminated in the contamination log and none of the tubes were excluded or were found to grow on the terminal blood-agar streaks. At 24 h, no bacterial or fungal overgrowth was seen in THP-1 infection wells. The observations described here are to monitor the formulations and do not form a formal sterility validation of the formulations.

3.10 Direct ingredient cost

Estimated direct ingredient cost per tube was US$0.0672 for G3, US$0.0254 for G2 (37.8% of G3), US$0.0285 for G5 (42.4%), US$0.0249 for G6 (37.1%), and US$0.0219 for G4/G8 (32.6%). G1 (US$0.0703; 104.6%) and G7 (US$0.0709; 105.5%) were slightly more expensive than G3, both of which contained the entire NNN solid phase, and also had some plant liquid. These estimates are based on ingredients only and do not reflect labour, consumables, equipment, quality assurance, storage or waste management.

Table 9. Estimated direct ingredient cost per culture tube (4 mL solid plus 4 mL liquid; US dollars, based on local prices).

Group	Composition	Cost per tube	% of G3
G1	NNN solid + plant liquid	0.0703	104.6
G2	Plant solid + ORS	0.0254	37.8
G3	NNN solid + ORS (control)	0.0672	100
G4, G8	Agar solid + ORS	0.0219	32.6

Group	Composition	Cost per tube	% of G3
G5	Plant solid + plant liquid	0.0285	42.4
G6	Agar solid + plant liquid	0.0249	37.1
G7	NNN solid + adjusted plant liquid	0.0709	105.5

4. Discussion

4.1 Principal findings

In this preliminary study, it was observed that the four-spice formulations tested were able to promote the growth of promastigotes identified as part of the L. donovani complex. In contrast, none of the formulations containing the plant was able to produce the same growth in promastigotes as the NNN-solid-plus-ORS control. G1 and G2 had an average AUC of around 77-84% and 86-87% of control, respectively, across three experiments. In experiment 1, the fully plant-based formulation G5 gave 67.9% of control AUC. The results indicate the need for further evaluation of selected plant-derived phases as alternative culture components to NNN, but do not prove equivalence or non-inferiority.

Only a little growth was seen in the plant-free agar–ORS and the unwashed-inoculum controls, indicating that the growth seen in the plant-containing groups was not simply from residual nutrients carried on with the inoculum or from agar, iron, gentamicin and ORS under the specified conditions. The growth-supporting environment created by the tested formulations is further supported by the control results. However, it does not specify which of the individual spices or constituents was the cause and does not rule out interactions between ingredients or differences in other medium ingredients. A blind second observer counted the cells, and an independent recount showed a mean absolute difference of 1.7%, and three isolates demonstrated the same trend. This consistency is limited in generalisability due to the small numbers of isolates and the lack of full lot crossing.

The other formulations help differentiate the performance of the two phases. G6 (agar with plant liquid) only gained 26.4% of the control AUC, suggesting that plant liquid alone was not capable of reproducing the growth support of the whole NNN-based control. The AUC was higher for replacement of the NNN solid phase with plant solid while keeping ORS (G2) than for replacement of the liquid phase with plant liquid (G1). The fully plant-based formulation (G5) did not work as well as G2. Both pH and osmolality were adjusted in G7, which resulted in an increase in AUC compared to G1. Still, it is not possible to distinguish whether this improvement was due to the pH adjustment, the osmolality adjustment, added sodium chloride or the combined effect of these adjustments. The results, therefore, indicate that the liquid phase properties are worth optimisation, and do not indicate a mechanism involving pH or osmolality.

Chemical data which are used to characterise the tested preparations do not give a mechanistic explanation of the obtained biological data. The measured soluble protein and reducing sugar concentrations of plant solid and plant liquid were lower than those of NNN solid. The folate was reduced after autoclaving, and the concentration of eugenol in the plant liquid obtained was less than the reported in vitro IC_{50} for L. donovani promastigotes [35]. The effects of the whole phytochemical mixture itself, other compounds and nutrient availability were not directly tested. The availability of heme and purines was not determined. Therefore, the increase in G2 and G5 growth cannot be linked to a specific nutrient or bioactive compound based on these observations.

The groups containing plants had poorer late culture results. The viability at day 20 was found to be lower in G1, G2 and G5 when compared to G3. Passage retention three passages was about 83 – 88

% for G1 and G2, while it was 95 – 99 % for G3. The effect on the outcome of pooled macrophage infection was also reduced for G1 and G2 compared to G3. The results show that the same level of early culture support does not always lead to the same level of late culture maintenance or culture infectivity. G5 was only evaluated for passage and infectivity in isolate 01, and the results are preliminary. The macrophage assay was also not used to examine intracellular survival or differentiation of the parasites later in infection, and infection was only evaluated at 24 hours.

From a cost point of view, G2 was the most favourable of the tested plant-containing formulations because the estimated cost of the ingredients in this formulation was US$0.0254 tube-1 (37.8% of G3), and the AUC was 86.9% of the control in experiment 1 and 85.9–86.9% across the three experiments. G5 also had a low ingredient cost (42.4% of G3) but only one isolate was used in the extended biological assessment, and AUC was 67.9% of the control. Cost estimates only include direct ingredients at local prices. They should not be seen as operational costs or as proof of cost effectiveness of routine diagnostic laboratories.

4.2 Research contribution and implications

A variety of other culture methods have been reported for Leishmania, including urine, preparations from lentils, bee pollen and serum- or blood-free media [15–23]. The present study also presents a preliminary evaluation of a specific four-spice mixture as a solid phase, liquid phase, or both phases in a biphasic system. The significance of this study is not that it demonstrates the ability of spices to substitute for the existing culture media, but that it provides a comparative study of phase substitution with plant-free and inoculum carry-over controls. Based on the results, the plant solid with ORS (G2) formulation was selected as the most promising formulation for further evaluation in the lab.

In addition to growth curves, the study also considers viability, serial passage, macrophage infection, medium chemistry, and direct ingredient cost. There are several questions that are still unanswered: the active or inhibitory constituent(s), the best mixture and concentration, the reproducibility between authenticated plant lots, and the performance across a fully crossed set of isolates and lots. The formulations have not been assessed for use in the isolation of clinical specimens, diagnostic sensitivity, drug-susceptibility testing, routine laboratory use, or long-term stability. The experiments should not be extrapolated to such applications.

4.3 Limitations

Several limitations should be considered. First, the three isolates were obtained from one source collection and were characterised as members of the L. donovani complex by ITS1-PCR and HaeIII digestion; sequencing and species-level confirmation were not performed. Second, botanical identity was based on supplier labels, with no voucher specimens or independent authentication. Only two plant lots and one mixture at one concentration were tested. Third, isolate and plant lot were not fully crossed, preventing separate estimation of isolate and lot effects. Fourth, the additional groups G5–G8, viability, culture-time pH, and final-medium chemistry were evaluated only in experiment 1, with four tubes per group. Passage and infectivity were assessed in three isolates for G1–G3, whereas G5 was assessed only in isolate 01. THP-1 passage numbers differed among runs (9, 11, and 13), and the source, catalogue number, and mycoplasma status of the THP-1 cells were not confirmed. Intracellular survival beyond 24 h and metacyclic proportions were not measured.

Other restrictions concern measurement and formulation. The pH of the culture was measured in individual satellite tubes, and the eight-group pairwise comparisons were based on pooled variance

estimates, despite small sample sizes and heterogeneity between the near zero and the growing groups. In the plant liquid, parasite-sized particles were present in the liquid, but the particle and promastigote spike recovery and morphology tests separated particles from promastigotes. The visible difference between plant liquid and ORS may also have limited effective blinding, even in the presence of coded tubes. After repeated sampling, the volume of liquid was decreased and the gentamicin levels in the NNN solid were different than in the other phases. The improvement in growth with the combined adjustment of pH and osmolality in G7 means that either of these variables cannot be singled out as being responsible for the improvement. Lastly, no diagnostic performance, clinical isolation, drug-susceptibility or storage-stability testing was conducted.

5. Conclusion

In this preliminary study, the tested four-spice formulation supported the in vitro growth of promastigotes that were reported as members of the L. donovani complex, both when used as a solid and as a liquid phase. The plant-free and unwashed-inoculum controls exhibited very little growth, suggesting there must be more to the observed growth than inoculum carry-over or non-plant components represented in those controls. However, growth in the plant-containing formulations was still lower than the growth in the NNN-based control, as the single-phase substitutions formulas yielded around 77-87% of the control AUC and the fully plant-based formula yielded 67.9% in experiment 1. Selected plant-containing groups also had less favourable late viability, passage retention and pooled macrophage-infection outcomes. The higher AUC in G7 cannot be assigned to either the pH or the osmolality adjustment independently.

The best growth/ingredient cost ratio was obtained with the plant solid with ORS (G2), which was estimated to be some 38% of the control, among the plant-containing formulations tested. No claims of equivalence to NNN or suitability for diagnosis, isolation from clinical specimens, drug-susceptibility testing or sustained routine maintenance can be made. Further work should use authentic plant material, longer serial passage, replicated infectivity testing with different isolates, additional lots and a fully crossed isolate x lot design, sequence-confirmed isolates, additional spice concentrations and combinations, and independently optimized pH and osmolality conditions. An operational cost analysis and validation for any planned laboratory application would also have to be performed.

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