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Original article

Preparation of a new culture medium by using *Ganoderma lucidum* for cultivation Of *Leishmania donovani* promastigote parasite

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ABSTRACT

Background: Leishmaniasis is a parasitic disease caused by the bite of infected sandflies carrying the *Leishmania* parasite. It's classified as a neglected tropical disease and affects many regions in the world, particularly in developing areas. **Aim:** *Ganoderma lucidum* powder is used to prepare a new culture medium for growing the *Leishmania* parasite in vitro, which consists of two stages. **Methods:** *Ganoderma lucidum* powder was used to prepare the solid phase instead of brain heart. This medium was attended by two groups. The first group prepared the solid phase of the powder of the *Ganoderma*, the second group prepared only the liquid phase was the filtrate of the *Ganoderma*. The third group was NNN media as a control group. **Results:** The parasite growth continued in a good way for a period of time. More than 20 days, with good vitality and natural shape, and without making a secondary subculture, increased numbers of parasites in the new *Ganoderma* powder medium, which was manufactured by adding *Ganoderma* powder to the solid phase, was a better increase than the medium in which *Ganoderma* powder was used in the manufacture of the liquid phase. Only its highest rate was on the eighth day, as it reached (54.5 and 48.7) x 10⁴ cells/ml, respectively, compared with NNN-media, which amounted to (58.4 x 10⁴ cells/ml), then the parasite preparation started decreasing with time until it became 20 days 0, 3 x 10⁴ cells/ml, respectively, while the NNN-media medium reached (6.5 x 10⁴) cells/ml, with significant differences between the two media. **Conclusion:** In both groups media, the results indicated an increase in the number of parasites counted over twenty days, with the highest increase occurring on the eighth day.

Introduction

The genus *Leishmania* causes Leishmaniasis is a disease caused by an obligate parasite. There are approximately 20 species

belonging to this genus that are transmitted to humans by a biological vector, which is an insect (female sand fly) *Phlebotomus* (with leishmaniasis in the Old World), and *Lutzomyia* (with leishmaniasis in the Old World). It has been proven

that there are approximately 30 species of this insect that transmit the disease [1]. Leishmaniasis is widespread in subtropical and tropical regions, with one million people infected with this disease annually [2]. Leishmaniasis appears in three clinical forms: Visceral leishmaniasis, called Kala azar, and the cause is *Leishmania donovani*, as this type is considered one of the most dangerous types. *Leishmania tropica* causes cutaneous leishmaniasis [3] and *Leishmania braziliensis* causes mucocutaneous leishmaniasis [4]. The three forms of infection range from infections without apparent symptoms to infections with severe symptoms [5]. The aim of this research was to obtain a commercially available and inexpensive material and use it instead of the materials used in culturing the *Leishmania* parasite, which requires special materials and conditions for culture and continued presence. Therefore, *Ganoderma lucidum* powder was used

The amastigote stage and the promastigote stage are the two stages in which this parasite exists. The amastigote stage is found in the human body, while the flagella phase is found in the transmitting insect (the sand fly). This is why the *Leishmania* genus is considered one of the parasites that need different hosts to complete its life cycle [6].

Given the medical importance of the leishmaniasis parasite, due to the symptoms it causes that may be fatal if left untreated, therefore, growing the parasite in vitro is important in diagnosis and treatment, in developing vaccines, and in developing chemical treatments for leishmaniasis. There are three main forms of media used to grow the *Leishmania* parasite: semi-solid media, biphasic media, and liquid media [7]. Semi-solid and biphasic media require the addition of blood, which is considered an important factor for parasite reproduction [8]. The process of isolating the promastigote phase of the *Leishmania* parasite from the culture media may sometimes be unsuccessful. This is due to several reasons related to the interaction of the parasite with the components present in the media. For this reason, many attempts have appeared to modify the culture media with various compounds to enhance the growth of the parasite on the one hand and increase its numbers on the other hand [9]. It has been noted that many of the essential compounds for the growth of the *Leishmania* parasite in agricultural media are available in the *Ganoderma lucidum*, as many studies conducted on the *Ganoderma lucidum* have

shown that 90% of the total weight of the fungus is water and it is rich in protein (10-40)% fat (2). -8)% Carbohydrates (3-28)% Fiber (3-32)% Minerals such as calcium, phosphorus, potassium, magnesium, iron, zinc and selenium. While other studies have indicated the presence of some steroid compounds, phenols, and polysaccharides, in addition to some important amino acids in this mushroom [10].

There are three basic types: liquid, semi-solid, and two-phase. Biphasic semi-solid media require blood as one of their components for parasite isolation, while fetal calf serum (FCS) for culture is added to liquid media [14]. Several types of media can be used for culture, including: M199 Medium, Nicolle-Medium, Evans' modified Tobie's Medium, RPMI 1640 Medium [11].

Previous studies have used milk from goats, cows, and buffaloes as a culture medium as a potential alternative to fetal bovine serum in cultures of visceral leishmaniasis. The results showed that 22 of 26 samples were positive for the protozoan flagellar stage (84.6%), and the cells were successfully maintained during the 6-month observation period. [19] In another study, crushed bovine liver and crushed bovine brain were used instead of brain heart infusion. A new culture medium was also prepared in which the Luc solution was replaced by a dextrolyte solution, and blood was added before sterilization (denatured blood). The same preparation method was followed in this study [21]. Other studies have used the addition of human urine to the culture medium to encourage parasite growth. [20]

Materials and methods

This study was conducted on a sample of *Leishmania* parasites obtained from the Department of Biology/Baghdad University. It was cultured in NNN medium and examined and confirmed under a light microscope at 40x magnification. It was then cultured in the *Ganoderma lucidum* medium. The statistical analysis was carried out by SPSS (v20).

Medium preparation

The first stage is solid: There are two methods of preparation

First method: consists of a solid phase and a liquid phase [10]. The materials are :Brain heart infusion 3.7 gm (BDH England), Dextrose 1 gm ((BDH England)), Agar 2 gm (BDH England) Blood 20 ml , Antibiotic 0.25 ml Gentamicin ((BDH

England)), Distilled Water 100 ml. First, sterilize the components at a pressure of one and a half atmospheres and a temperature of 121 degrees Celsius in an autoclave for 15 minutes. For the purpose of cooling the medium, place it in a water bath at a temperature of 50-55 degrees Celsius. Then the antibiotic is added, and at a rate of 5 ml of the medium it is distributed in containers. Sterile glass with a tightly closed metal lid. Leave it until it solidifies completely, and incubate it at a temperature of 37 degrees Celsius for a full day to ensure its effectiveness by being free of contamination. Then the containers are placed in the refrigerator at a temperature of 4 degrees Celsius.

The second method: Preparation of the medium with the replacement of Brain heart infusion and Dextrose [3] ; The solid phase: The materials are: *Ganoderma lucidum* 4.7 gm (Organo Gold USA), Agar agar 2 gm (BDH England), Blood (From rabbits) 20 ml, Antibiotic Gentamicin 0.25 ml (BDH England), Distilled Water 100 ml.

Sterilize all materials at a pressure of one and a half atmospheres and a temperature of 121 degrees Celsius in an autoclave for a period of not less than 15 minutes. For the purpose of cooling the medium, the medium is placed at a temperature of 50-55 degrees Celsius in a water bath, then finally the antibiotic is added, and this medium is distributed at a rate 5 ml of it in sterile glass containers with a tightly closed metal lid and leave it until it solidifies completely, and incubate it at a temperature of 37 degrees Celsius for a whole day, i.e. 24 hours, to ensure its effectiveness by being free of contamination, then put it in the refrigerator at a temperature of 4 degrees Celsius [7].

The liquid phase

First: In the preparation, oral rehydration salts were used, which is given to children with diarrhea, and their components (BDH England) are: potassium chloride 1.5 g, sodium chloride 2.6 g, anhydrous glucose 13.5 g, sodium citrate 2.9 g. In (1 liter), all contents were dissolved in distilled water, and at a temperature of 121 C, they were sterilized in an autoclave at a pressure of 1.5 atmospheres for 20 minutes, and finally an antibiotic was added to them, and they were placed in sterile bottles and placed at 4 C in the refrigerator.

Second: Weigh 2.5 grams of *Ganoderma lucidum* powder and dissolve it in 100 ml. Using filter papers, it is filtered to get rid of undissolved

particles, the filtration process is performed by pouring the mixture onto a porous filter paper. The porous filter paper must be placed in a funnel. 602H grade filter paper has a pore size of 2 micrometers. This type of filter paper has the smallest pore size of all standard quality filter papers. It is used to collect or remove fine particles. Then, the filtrate is taken, In an incubator at a temperature of 121 degrees Celsius, it is sterilized at a pressure of 1.5 atmospheres for 20 minutes. Finally, the antibiotic is added to it, kept in the refrigerator and placed in sterile bottles at a temperature of 4 degrees Celsius [7].

Leishmania donovani parasite

The parasite was obtained from Department of Biology/Baghdad University and cultivated on NNN-medium and the *Leishmania donovani* was in a Promastigote phase.

Parasite Cultivation

Leishmania parasites are cultured at the initial inoculation concentration (1×10^3 cells/ml) in media at a rate of (0.5 ml) to achieve several objectives: First, this concentration ensures that there are a sufficient number of parasite cells to establish infection in the media. Second, this concentration allows the parasite to be studied more precisely in media [22]:

1-G1: The parasite was cultured in 4 vials containing a medium containing (4.7 g) of *Ganoderma lucidum* powder + Agar in the solid phase (5 ml) and the liquid phase was a Lock solution.

2- G2: The parasite was cultured in 4 vials containing NNN medium, and the liquid phase was filtrate of *Ganoderma lucidum* powder (5 ml in each tube).

3- G3: The parasite was cultured in 4 vials containing NNN medium, as a control group.

After (2, 4, 6, 8, 10, 12, 14, 16, 18, 20) days, the numbers of parasites were counted using a hemocytometer in the culture, and they were examined using an optical microscope under a lens (40x).

Ethical consideration

The written consent obtained of Biology/Baghdad University for obtain of leishmania parasite strain.

Results

Ganoderma lucidum powder, which is available in local markets at simple and inexpensive

prices, was a new cultural medium used to prepare for development of the anterior flagellate stage of the parasite, *Leishmania donovani*. The medium consists of the first and second parts, The upper part is the liquid phase and the lower part is the solid phase. *Ganoderma lucidum* powder was added as a basic material in preparing the solid medium with the addition of denatured blood and agar. As for the upper part, the solution of oral irrigation was taken as an alternative to Luke's solution, and *Ganoderma lucidum* powder was also added to it. For the purpose of knowing the extent of the efficiency of this medium, it was compared with the medium used internationally, which is considered One of the cheap and efficient media is NNN-media, and the results were good and close to the results of NNN-media. The parasite continued to grow well for more than 20 days, with good vitality and its natural appearance, without any secondary culture (subculture). After (2, 4, 6, 8, 10, 12, 14, 16, 18, 20) days, the numbers of parasites were counted, and the results are in Table (1): The highest increase in the numbers of parasites in both media was reached on the eighth day. In the medium of *Ganoderma lucidum* powder, an increase in the number of

parasites was observed, which was manufactured by adding it to the solid phase, and the *Ganoderma lucidum* powder filtrate was used instead of Luke's solutions, was better than the rest of the media in which the *Ganoderma lucidum* powder was used in manufacturing The liquid phase only, as a substitute for Luke's solution, reached its highest rate on the eighth day, reaching $(54.5 \text{ and } 48.7) \times 10^4$ cells/ml, respectively, compared to NNN media, which reached 58.4×10^4 cells/ml. The number of parasites began to decrease after 20 days. $0, 3 \times 10^4$ cells/ml, respectively. While the NNN media reached 6.5×10^4 cells/ml, it is observed that there are no significant differences.

Statistical analysis

The statistical analysis was carried out by SPSS (v 20). ANOVA test was used to analyses repeated measure between tests concentration and control. Data expressed as mean $\pm$ SD and values of ($p > 0.05$) were considered statically non-significant while ($p < 0.05$) and ($p < 0.01$) were considered significantly different, highly significantly different respectively LSD test was used to calculate the significant differences between tests mean.

Table 1. Media at times to calculate average numbers of parasites per milliliter.

P VALUE	G1: Liquid media	G2: Solid media	G3: NNN media	DAY
0.05	G 20.5 $\pm$ 0.5	J 17.75 $\pm$ 0.5	H 18.5 $\pm$ 0.5	2
0.05	D 36 $\pm$ 0.8	G 28.5 $\pm$ 0.5	F 31.2 $\pm$ 0.8	4
NS	C 40.81 $\pm$ 0.5	D 45 $\pm$ 0.7	D 44.5 $\pm$ 0.5	6
0.05	A 48.7 $\pm$ 0.5	A 54.5 $\pm$ 0.5	A 58.4 $\pm$ 0.5	8
NS	C 41.5 $\pm$ 0.4	B 50 $\pm$ 0.3	B 51.5 $\pm$ 0.3	10
0.05	E 33.5 $\pm$ 0.5	E 41.76 $\pm$ 0.5	D 44.5 $\pm$ 0.5	12
NS	F 28.55 $\pm$ 0.5	G 28.4 $\pm$ 0.3	F 31.65 $\pm$ 0.5	14
NS	G 18.7 $\pm$ 0.8	I 20.4 $\pm$ 0.3	H 21.5 $\pm$ 0.5	16
0.05	-----	L 9.5 $\pm$ 0.5	I 15 $\pm$ 0.3	18
	-----	3 $\pm$ 0.5	6.5 $\pm$ 0.5	20
P VALUE	0.0001	0.0001	0.0001	
LSD	3.41	2.9	3.3	
Results were expressed as mean+ standard error (SE) and values of $p > 0.05$ were considered Statically non significant while $p < 0.05$ and < 0.01 , < 0.001 , < 0.000 were considered significantly different ,highly significantly different respectively . LSD test was used to calculate the significant differences between the tested mean , the letters (A,B,C,D,E, F,G,H and I) for column represent the levels of significance, highly significant start from the letter A and decreasing with the last one . Similar letters mean there are no significant differences between tests mean.				

Discussion

For the purpose of useful scientific research, the *Leishmania* genus in the laboratory (*in vitro*) is a useful way to produce a large quantity of parasites for diagnostic purposes to provide more information that will benefit us in the future about

the relationships between the host and the parasite and to determine the biological and immunological characteristics of the parasite. One of the most important goals of *Leishmania* multiplication is to maintain efficient and divided populations of its various types. The different media can be classified

into two main classes: semi-solid biphasic media and liquid monophasic media. *Leishmania* promastigotes were first grown on diphasic blood agar (NNN) [12]. Heart-brain infusion is added to the medium and used with various modifications from the liquid phase added to the solid phase [13]. To adapt and culture *Leishmania* strains immediately separated from different vertebrate and invertebrate species, these biphasic media are used, which have not been identified until today [14]. The host generally used is Brain Heart Infusion with partial serum. This revealed the risk of host contamination of the recombinant product [15]. *Ganoderma lucidum* is a mushroom in red, light yellow, or brownish-red colors and is used in Far East countries especially Japan, China, and Korea for pharmaceutical purposes [16]. Its fruit body and mycelia contain different groups of compounds including sterols, steroids, peptides, lactones, alkaloids, polysaccharides, triterpenoids, and proteins with proven medicinal effects [18].

In this study, it was found that it was possible to use *Ganoderma lucidum* powder in the manufacture of a new, highly efficient medium for parasite growth, and that adding denatured blood enhanced the continuity of the increase in the parasite's numbers and the preservation of its vitality for a period of 20 days, the culture is not renewed because there is no need because the natural form of the parasite remains. *Ganoderma lucidum* powder medium has high efficiency and low contamination compared to other media. This may be due to several reasons, including that the use of denatured blood led to the breakdown of blood cells and its use became easier. By parasite. It was observed through experience that the oral irrigation solution was less susceptible to contamination than the oral irrigation solution. The results confirmed that the *Ganoderma lucidum* powder filtrate can be used as an alternative to Luke's solution because it contains many elements necessary for the growth of leishmaniasis, the most important of which are carbohydrates, proteins, and fats found in the mushrooms without the need to re-cultivate them every 8 days, while the parasite retains its vitality [7].

The flagellated phase needs nutrients, stimulating compounds, and appropriate environmental conditions in order to grow, and these nutrients include sugar, which is a source of carbon, proteins, which are a source of amino acids, and fats, which are a source of energy [17].

For maintaining and cultivating *Leishmania* parasites, most culture media are supplemented with FBS, also known as fetal calf serum (FCS). FBS is highly favored due to its rich content of growth-promoting factors, proving crucial for mass culturing of various *Leishmania* species and strains [22].

In vitro, protein content and *Leishmania* promastigote growth rate are closely related, with protein availability and the type of proteins influencing growth. *Leishmania* promastigotes can be cultured in media supplemented with proteins, like fetal bovine serum (FBS), which supports their growth and development. The specific proteins present in the culture medium, along with the parasite's own protein metabolism, significantly impact the overall growth rate and differentiation of *Leishmania* promastigotes, *Ganoderma lucidum* powder rich with protein it contains (10-40)% [10].

Higher glucose concentrations generally lead to increased growth rates, while glucose limitation can reduce growth. The specific rate of glucose consumption by promastigotes also depends on the growth rate and availability of glucose. Nevertheless, previous research also showed a positive correlation between the glucose availability in the culture medium and *in vitro* promastigote growth for *Leishmania* [23].

Conclusion

The *Ganoderma lucidum* powder could be used as a cost-effective Medium for culturing *Leishmania* promastigotes. and was uncomplicated, simple and fast and did not require the addition of other substances such as serum. Moreover, the preservation of the parasite in its form and vitality indicates the availability of substances important for growth.

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Ethical consideration

The written consent obtained of Biology/Baghdad University for obtain of *leishmania* parasite strain.

Conflict of interest

The authors declare that there is no conflict of interest.

Data availability

In this research, the focus is on the accessibility, reliability, and timeliness of the data. Data redundancy and backup play a role in maintaining high data availability for future research.

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Authors' contribution

The authorship of the title above certify that they have participated in different roles as follows: **Sabaa Taher Mohammed**: Conception, design and preparing the media. **Hanan Faisal Ghazi**: Preparing the media for culture. **Haider Yousif Ahmed**: Recalculating numbers in culture media and compare the numbers with the control. **Hadeel Abdulatif Majeed**: Calculate the numbers in the culture medium. **Hamzia Ali Ajah**: Compare the numbers with the control medium. **Ekhlass N.Ali**: Recalculating numbers in culture media. **Luma Qasim Ali** : Organizing and analyzing data.

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