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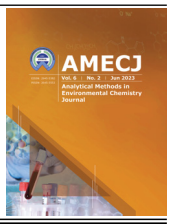

Gas chromatography analysis of plant extracts to examine ingredients: Turmeric extracts on Leishmania Promastigotes and anti-Leishmania effect of Ginger

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ABSTRACT

Turmeric extract and aroma oil of *Curcuma longa* exhibit inhibition properties against various bacteria, parasites, and pathogenic fungi. We investigated the effects of Ginger (*Zingiber officinale*) and Turmeric extract on *Leishmania* promastigotes and used gas chromatography-mass spectrometry (GC-MS) for analyzing plant extracts. The hydroalcoholic extractions obtained from the two plants were diluted in 70% ethanol to three different concentrations; 12.5, 100, and 500 mg mL^{-1}. The *Leishmania significant* strains were propagated in an artificial medium to reach sufficient parasites. The survival percentage of *Leishmania* promastigotes was affected significantly by the time and concentration of the extracts ($P < 0.05$). The repeated measures pattern showed an interaction effect between various time points and treatment with the extracts. statistics analysis showed a significant difference between different concentrations and extract samples ($P < 0.05$). GC-MS showed that the survival rate of *Leishmania* promastigotes treated with hydroalcoholic extract of Ginger at 3-time points (24, 48, and 72 hours) was lower than Glucantime and Turmeric extract. The survival rate of promastigotes treated with Turmeric extract was similar to those treated with Glucantime but lower than those treated with a combined extract of Ginger and Turmeric at a concentration of 500 mg mL^{-1}. The 50% inhibitory concentration (IC50) values of Ginger and Turmeric plant extracts were similar to that of Glucantime, indicating that these extracts can be used as potential drug candidates for leishmaniasis.

1. Introduction

Herbal extracts and medicines are still actively used to treat various diseases [1]. Ginger (*Zingiber officinale*) is a famous spice in East and Southern Asia and other parts of the world. About 100,000 tons of Ginger are produced annually, of which 80% is made in China. Ginger has long been used to treat musculoskeletal diseases due to its anti-inflammatory properties [2]. Many

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literature reviews have been *published* on the mechanisms of action of medicinal Ginger. For example, the study of Grzanna [3] concerned using Ginger as an anti-inflammatory factor. At the same time, the literature of Shukla and Singh [4] examined the cancer-prevention properties of this raw drug. In addition, Chayakonaprok [5] investigated the mechanisms of the effect of Ginger as an anti-emetic after surgery. Many researchers have recently surveyed in vivo and in vitro the effects of anti-parasite essential oils and some of their extracts from native plants. *Zingiber officinale has* antifungal, antibacterial, and antiparasitic properties [6-9]. The identified ingredients of *Zingiber officinale* consisted of gingerols, shogaols, 3-dihydroshogaols, paradols, dihydroparadols and derivatives of acetyl gingerols, gingerdiols and isolated mono and diacetyl gingerdiols, 1-dehydrogenadiones, diarylheptanoids, and methyl Combinations [10]. The Zingiberaceae family consists of more than 80 species, of rhizomatous and herbaceous plants; *Curcuma* is one of the Plants of this family [11]. *Curcuma longa* is a rhizomatous flowering plant widely studied and has excellent potential for effectively treating many diseases [12]. *Curcuma longa* L. (*Zingiberaceae*) is an herbaceous plant native to tropical regions of Asia. It is commonly used as a spice to add flavor to food and impart color. The powdered form, called turmeric, it is also used for medicinal purposes [13]. Curcumin is a polyphenol, non-toxic and medicinal, antioxidant, active anti-inflammatory ingredient, and antiparasitic activities [14]. Antiprotozoal properties of *Curcumin* have also been discovered both in vitro and in vivo for *Plasmodium* [15], *Leishmani*a [16-18], *Trypanosoma* [19], and *Giardia lamblia* [20]. A recent study has also shown that it works against promastigote forms of *Leishmania major* [21]. Curcumin exerts its anti-inflammatory activity by inhibiting several important inflammatory molecules. Turmeric powder is effective in reducing inflammation after surgery. It also helps to prevent atherosclerosis by reducing clot formation in blood vessels [22]. Turmeric extract and essential oil of *Curcuma longa* exhibit inhibitory effects against various bacteria, parasites, and pathogenic fungi. In an animal study, guinea pig models of dermatophytes, pathogenic molds, and yeast were treated with topically applied turmeric oil or curcumin. The researchers indicated that dermatophytes and pathogenic fungi were inhibited by turmeric oil, but the yeast isolation was neither affected by curcumin nor turmeric oil. Dermatophyte and pathogenic mold lesions induced on the guinea pigs improved with treatment and disappeared seven days' post-turmeric application. Curcumin exhibits moderate anti-*plasmodium falciparum* and anti-*Leishmania major* effects [22]. Curcumin was found to be responsible for most of these cases' biological effects of turmeric [23]. Curcumin has anti-leishmania activity in laboratory conditions [17]. Several researchers have found the action of curcuminoids isolated from *C.longa* against *Leishmania major* with IC50 values from 22 to 60 M [17, 18, and 24].

Therefore, this study evaluated the effectiveness of *Zingiber officinale* and *Curcuma longa* extracts on the inhibition of promastigotes of *Leishmania* in vitro.

Plant chromatographic analysis can be done using plant extracts determined by GC-MS, HPLC, HPLC-MS, and GC-FID. The pharmaceutical industry uses the HPLC method, preparative and analytical techniques to separate and purify herbal compounds. HPLC-MS Spectroscopy is a high-performance liquid chromatography that is very useful for qualitative analysis. Due to the equipment's high sensitivity, stability, and efficiency, GC and GC-Ms equipment are accepted methods for studying volatile components of herbal medicines. GC-FID, several detectors are used for gas chromatography. GC-FID has a high initial sensitivity to hydrocarbons [25].

The current project aimed to evaluate the effects of this plant's extracts on Leishmania promastigotes and used gas chromatography (GC-MS) and mass spectrometry to analyze plant extracts.

2. Material and method

2.1. Plant Specimens and Preparation of Hydroalcoholic Extractions

Dried rhizomes of ginger and turmeric were obtained from the herbal store. The dried rhizomes were pulverized entirely with a hand mortar and filtered or passed through a No. 10 sieve. To prepare hydroalcoholic extractions**,** the powdered form of the rhizomes was mixed with 70% ethanol in a ratio of 1:1. In addition; hydroalcoholic extracts were obtained by mixing for a week in the shaker incubator. Then, the extracts were then dried in an oven at 40°C to remove the alcohol. Hydroalcoholic *extracts* obtained from the two plants were diluted in 70% ethanol in three different concentrations; 12.5, 100, and 500 mg mL^{-1}. The solvent was mixed with the extract and sterilized by filtration. The final concentrations of 12.5, 100, and 500 mg mL^{-1} of all extracts were prepared in 1 mL of culture medium.

2.2. Preparation of parasite culture

Parasites were cultured in an NNN medium in laboratory conditions. Following culture in the NNN medium, the *L. major* strains were transferred to the RPMI medium. The *L. major* strains were initially propagated in an artificial medium (RPMI 1640 with 10 - 20% FBS) to reach sufficient parasites. The anti-leishmania agents of the extracts were investigated in the stationary phase of the growth curve of promastigotes.

2.3. Cytotoxic effects of hydroalcoholic extracts and cytotoxicity Test

Promastigote cells were collected at the stationary phase and numbered using a cell counter. To evaluate the anti-*Leishmania* effects of Ginger and *Curcuma longa* extractions in RPMI 1640 culture medium, concentrations of 500, 100, and 12.5 mg mL^{-1} were applied. 10 µL of each dilution series made was added to an Eppendorf tube. The dilution used to prepare the hydroalcoholic extract was 70% ethanol. The solvent is blended with the extract in small amounts. The inhibitory effect of each dilution of the extract on *Leishmania* was determined in three 1.5 ml Eppendorf tubes. 80 µL of parasite suspension was added to 20 µl of diluted ethanol for negative control. Glucan time in 12.5, 100, and 500 mg mL^{-1} concentrations of such positive control were used. After the initial parasites count, the plates were re-incubated at a temperature of 27°C, and the number of parasites in 10 µL for three days was counted by Neobar slide. Eventually, the mean number of promastigotes in all three plate series and the percentage of live parasites were calculated for each concentration.

For the cytotoxicity test, 100 µL of parasite suspension (1×10^6 Promstigote per mL) was cultured in an incubator at 27°C for 72 h in a 50 mL vial. The cell suspension was placed in two rows of wells (A-H) in a Nunc-Immuno™ 96-well microliters plate (Maxi Surf™ surface). After aspiration of the medium, 150 µL of the highest concentration of the Ginger and *Curcuma longa* extracts was added into the rows in a serial dilution. Wells containing only parasite cell suspension without any extract were used as controls. 10 µL of M.T.T reagent (3-4, 5-dimethgylthiaol-2-yl-2, 5-diphenyltetrazolium bromide) was added to each well and we incubated. After the withdrawal of the medium and M.T.T reagent, dimethyl sulfoxide (DMSO) (100 µL) was added to the plates and stirred for 5 min. The absorbance of each well was measured using a micro titer plate reader at a wavelength of 490 nm [26].

2.4. Gas Chromatography Mass Spectrometry Analysis (GC-MS)

Gas chromatography-mass spectrometry analyzer (GC-MS) was used to analyze and identify the compounds of essential oregano oil (Hewlett-Packard 6890, Agilent Technology, and Santa Clara, CA, USA) and equipped with HP-5MS column (30m×0.25mm×0.25µm). The primary temperature used was 40 °C for 1 min and later increased to 220 °C at a rate of 3 °C per minute and finally raised to 270 °C for 5 min at a rate of 20 °C per minute. Other GC-MC instrument parameters include helium carrier gas (999.99%), injector temperature (260°C), detector temperature (FID, 270°C), split

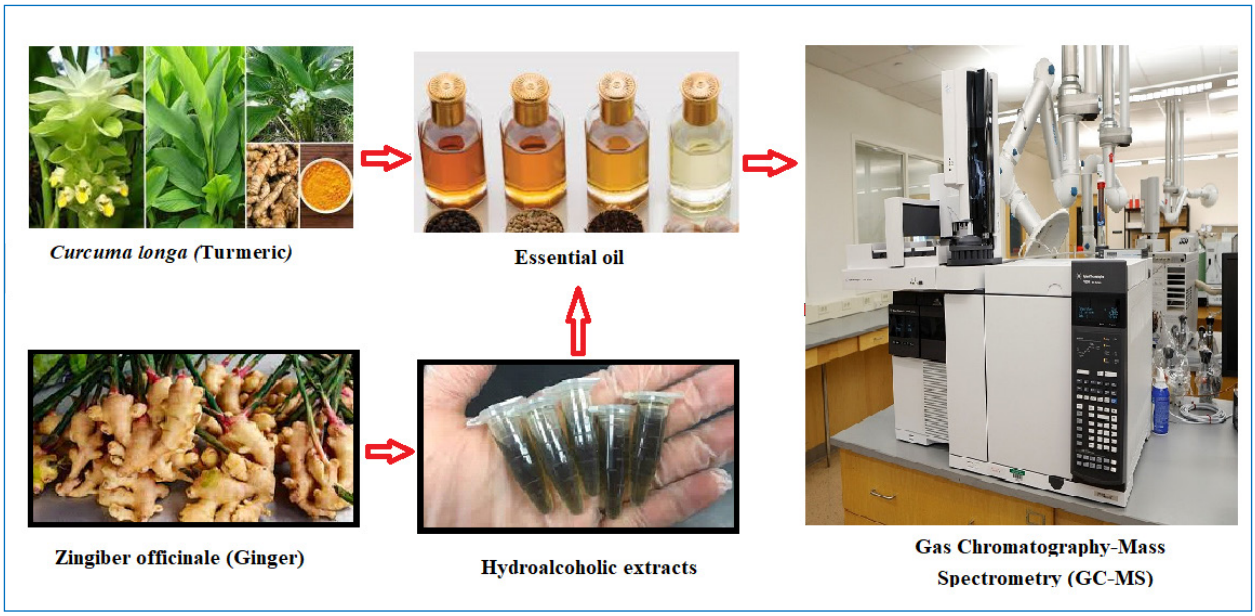

Fig.1. Different stages of plant collection, extraction of hydroalcoholic extract of plants and detection by the device GC-MS.

less mode, and potential ionization 70eV, scan rate, a scan speed of 1 scan per second, scan range m/z 40-48 was used for all analyses. Essential oil compounds were identified by comparing their retention indices, and fragmentation mass spectra with those stored in a Wiley 7n.1 mass computer library and the National Institute of standards and Technology (NIST) [27, 28]. The research stage is illustrated in Figure 1.

2.5. statistical Test Analysis

Data analysis was done using SPSS version 22 software. Repeated measurement tests and analysis of variance tests were used to compare the data. Average survival and the percentage of promastigotes in different concentrations of extract and times were analyzed using the Post Hoc test and Tukey's multiple comparison test. statistical significance was discussed when $P \leq 0.05$ was significant.

3. Results and Discussion

3.1. Growth responses of the parasite to extracts at different concentrations and time Point

In the present study, the anti-*Leishmania* effects of Ginger and Turmeric were investigated under laboratory conditions and were demonstrated and compared with Glucantime. One-way ANOVA analysis showed that ginger and turmeric extracts significantly affected the survival percentage of *Leishmania* ($P < 0.05$) at different concentrations and time points (Table 1). In addition, no significant differences in viability percentage of *Leishmania* were observed when treatment with the two plant extracts was compared with Glucantime (Table 1). Interaction effects between different time points and extractions were observed when the repeated measures test was performed. The repeated measures test also, a significant difference was showed between various concentrations and kinds of extracts ($P < 0.05$) and between extracts at different time points and concentrations ($P < 0.05$) (Table 1). Tests for comprising the mean (Tukey's multiple comparison test) was used to estimate the average survival percentage of promastigotes of *L. major* in various concentrations of two plant extracts for both extracts, a concentration of 500 mg mL^{-1} was the most effective in killing promastigotes ($P < 0.05$). Tukey's mean comparison test showed no significant difference between the viable percentage of promastigotes in 24 hours, 48 hours and 72 hours (Table 2a, 2b, 2c).

Table1. In-vitro effects of Ginger *(Zingiber officinale)* and Turmeric *(Curcuma longa)* extracts on the viability percentage of *Leishmania* promastigotes at different time points and concentrations.

variables		Sum of squares	df	Mean square	**F**	**Sig.**
Percent of viability -24h	Between groups Within groups Total	8455.233 1502.785 9958.018	3 8 11	2818.411 187.848	15.004	0.001
Percent of viability -48h	Between groups Within groups Total	9424.207 1536.474 10960.68	3 8 11	3141.402 192.059	16.356	0.001
Percent of viability -72h	Between groups Within groups Total	8714.994 1700.643 10415.64	3 8 11	2904.998 212.580	13.665	0.002

Table 2a. Comparison of the mean percent viable *Leishmania* promastigotes at 24 h for the different plant extract points (Tukey HSD test).

Groups	**Time**		
	24h	**48h**	**72h**
Glucantime	28.0033	-----	-----
Curcuma longa	58.1933	58.1933	-----
Zingiber officinale	45.5667	-----	-----
control	-----	-----	100.0000
Sig.	0.257	0.102	1.000

Table 2b. Comparison of the mean percent viable *Leishmania* promastigotes at 48 h for the different plant extract points (Tukey HSD test).

Groups	**Time**	
	24h	**48h**
Glucantime	29.0900	-----
Curcuma longa	34.1933	-----
Zingiber officinale	58.6133	-----
control	-----	100.0000
Sig.	0.115	1.000

Means for groups in homogenous subsets are displayed
Subset for alpha=0.05

Table 2c. Comparison of the mean percent viable *Leishmania* promastigotes at 72 h for the different plant extract points (Tukey HSD test).

Groups	**Time**		
	24h	**48h**	**72h**
Glucantime	30.1000	-----	-----
Curcuma longa	42.9100	42.9100	-----
Zingiber officinale	-----	71.0967	71.0967
control	-----		100.0000
Sig.	0.713	0.161	0.149

Means for groups in homogenous subsets are displayed
Subset for alpha=0.05

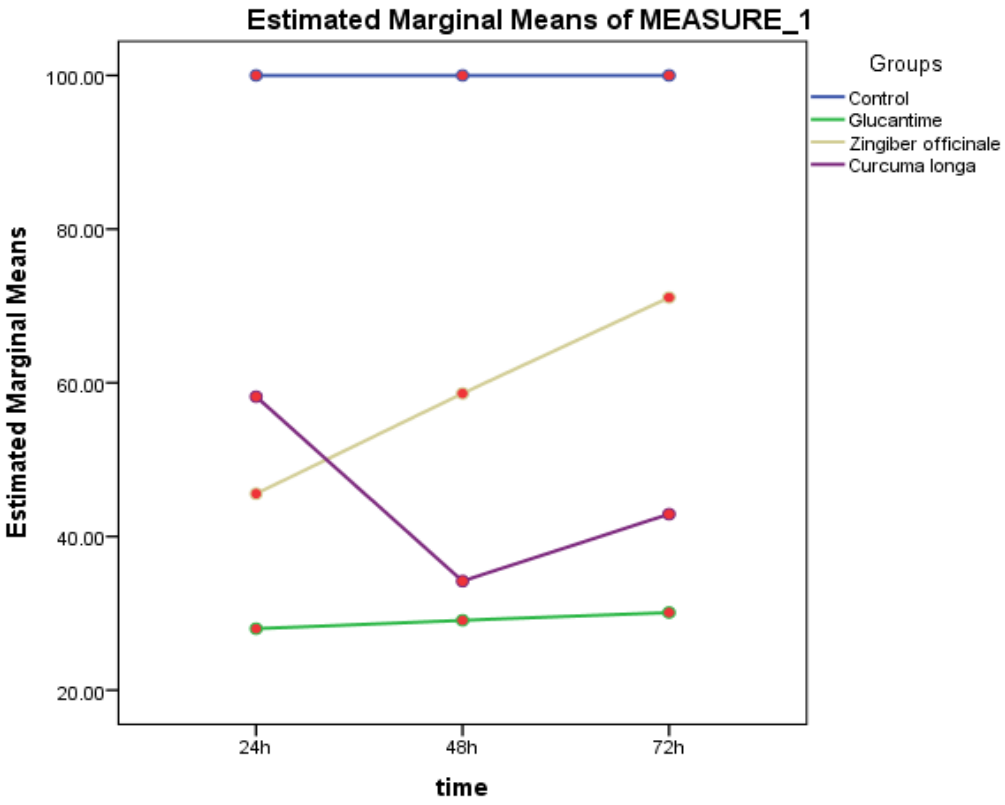

Fig. 2. Viability of *Leishmania major* promastigotes (MRHO/IR/75/ER) at different exposure times against hydroalcoholic extracts of Ginger, Turmeric, and Glucantime. Each point represents the average of three independent tests.

3.2. Analysis of the effects of extracts on live Promastigotes of Leishmania major by M.T.T assay

The results in Figure 2 show that the survival rate of *Leishmania* promastigotes treated with hydroalcoholic extract of ginger at 3 time points (24, 48, 72 hours) was lower than Glucantime (positive control) and turmeric. The survival rate of promastigotes treated with turmeric extract was similar to Glucantime but lower than those treated with a combined extract of ginger and *Curcuma longa* at a concentration of 500 mg mL^{-1}.

3.3. Sort by Mortality

Sort by mortality followed by Ginger › Glucantime › *Curcuma longa* › Ginger 500 mg mL^{-1} + *Curcuma longa* 500 mg mL^{-1}.

3.4. Analysis of plant extracts by gas chromatography and mass spectrometry

Ginger extract

The chromatogram obtained from the study of the ginger hydroalcoholic extract is shown in (Fig. 3). Gas chromatography identified 15 compounds with specific frequencies that are listed in (Table 3). Gas chromatography identified 15 bioactive compounds corresponding to the peaks shown in (Fig.3). Among the compounds, 1, 2-Dithiacyclohexane (30.107%) was the most abundant with a retention time of 40.008. It is present in the bites of insects such as ants and bees and is also the main compound in nettle leaves [29]. Among the bioactive compounds, 1, 2-Dithiacyclohexane was the most abundant 30.107% with a retention time 40. 008. This compound is present

in insect venom, especially in the venom of ants and bees. It is also a bioactive constituent of nettle leaves. The oil constituent Telfairic acid was also identified with a relative abundance of 20.312% and a retention time of 34.591. This compound is lipid in nature. Other important bioactive compounds identified in the extract include the phenolic compound 2-methoxy-4-2-propenyle, with a relative abundance of 2.957% and a retention time of 43.112. This compound has known anti-microbial and antioxidant properties. Also, 2, 3, 4, and 5-tetramethyl thiophene 1.61% is another phenolic compound identified in the extract with anticancer properties.

3.5. Curcuma longa extract

The chromatogram obtained from the study of turmeric hydroalcoholic extract is shown in Figure 4. Based on the result, 20 compounds were identified and are listed in (Table 4). Z, Z-3, 9-Cis-6, and 7-epoxy-nonadecadiene were the most

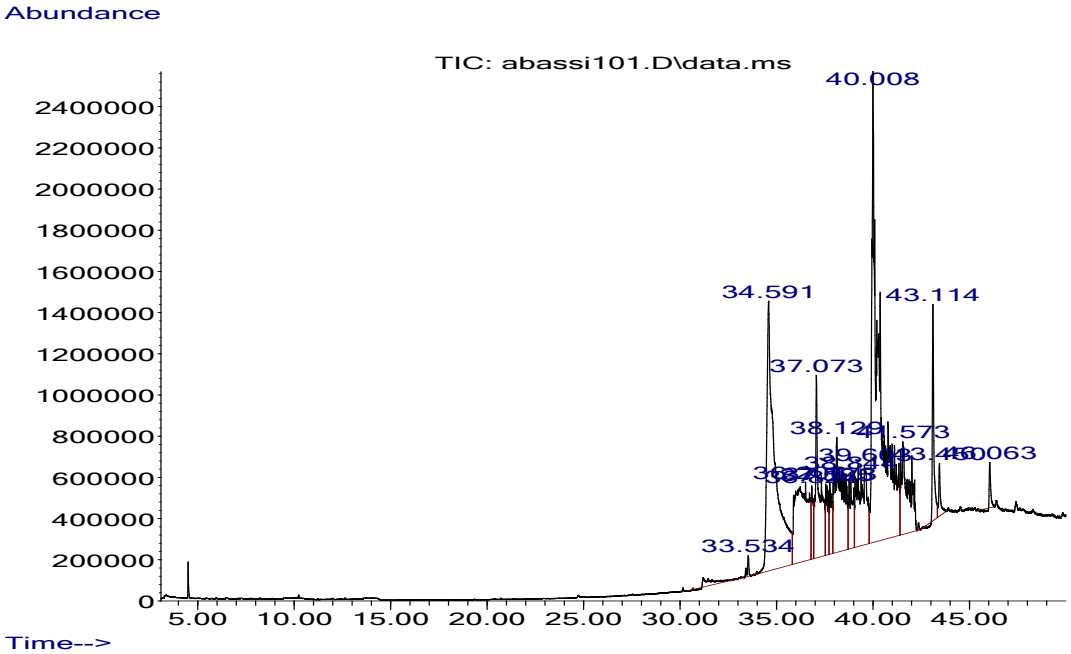

Fig. 3. Gas chromatographic-MSanalysis of Ginger extract.

Table 3. Ginger extract compounds

Compound	RT	%Area
9,12,15-Octadecatrienoic acid, methyl ester	33.536	1.055
Telfairic acid $$ Grape seed oil	34.591	20.312
2-Methyl-z,z 3,13-octadecadienoic	36.205	9.602
Cyclododecyne	36.823	1.266
Phenol,2-methoxy-(CAS)	37.074	6.655
2-Naphthol,1-(p-chlorophenyLazo)	37.592	1.652
2,3,4,5-tetramethyl thiophene	37.773	1.61
N-Methyl-3,3-dimethyl-2-hydroxy butanoic acid amide	38.129	8.16
Cyclooctene,3-ethenyl	38.845	2.595
Methoxy-4-vinyl phenol	39.603	6.44
1,2-Dithia Cyclohexane	40.005	30.107
Bicyclo[10.1.0]tridec-1-ene	41.573	6.182
Phenol,2-methoxy-4-(2-prophenyl)-CAS	43.112	2.957
Vanillin	43.45	0.889
Benzene acetic acid,4-hydroxy-3-methoxy	46.06	0.518

numerous compounds found in the extract with a relative abundance of 20.082 % and a retention time of 40. 011. It is an organic aldehyde with medicinal and therapeutic properties. Tridecanedial 5.955% is a Trichloroethylene compound used as an industrial solvent. Its use in the food and drug industry was banned in several countries in the 1970s due to its toxicity National research, 2007. The hydrocarbon compound Tetradecyl belonging to the Alkyne group, was also identified in the extract. Eicosene 5.786 % is another hydrocarbon compound identified in he extract.

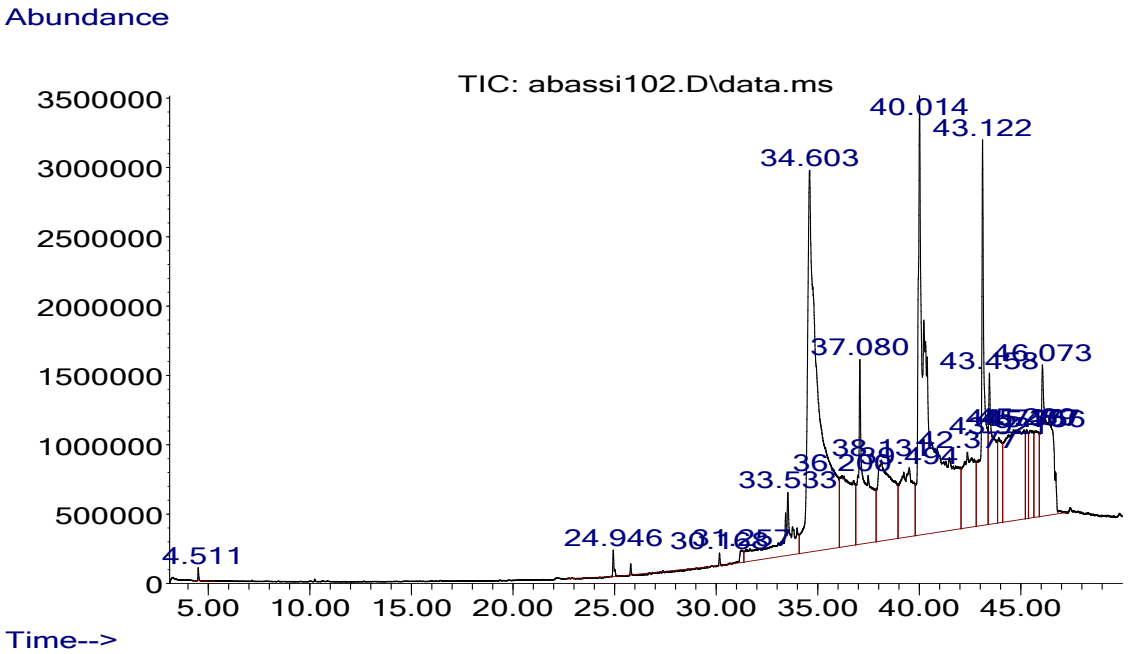

Fig. 4. Gas chromatographic analysis of *Curcuma longa*.

Table 4. *Curcuma longa* extract compounds

Compound	RT	%Area
3-METHYL BUTYL ACETATE	4.509	0.053
Benzen,1-methyl-3-(1-methylethyl)	24.944	0.084
Hexadecanoic acid, methyl ester	30.167	-0.189
Thiosulfuric acid ($H_2S_2o_3$),	31.257	0.23
6-Octadecenoic acid, methyl ester(CAS)$$Methyl 6-octadecenoate	33.53	3.433
5-Tetradecyne$$5C14H26	34.602	21.01
Curcumin Keto from(C15H20O)	36.199	4.458
9-Eicosene,(E)	37.08	5.786
10-Undecenoic acid (CAS)	38.129	5.819
Oleine 7503	39.493	4.15
Z, Z-3, 9-Cis-6, 7-epoxy-nonadecadiene	40.011	20.082
Tridecanedial	43.124	5.956
Trifluoroacetoxy hexadecane	43.456	3.745
Trans 12-Octadecadienoate	43.922	1.741
Bicyclo[10.1.0]tridec-1-ene	44738	8.09
E-11,13-Dimethyl-12-tetradecane 1-OL acetate	45.286	1.142
2-hydroxy-1-(hydroxymethyl)ethyl ester	45.479	1.913
7-pentadecyne	47.758	1.912
1,3 Cyclohexadecanedione	46.073	6.422

3.6. Discussion

Many studies have been done on the effect of herbs such as Thyme, Yarrow, Propolis, Siderite, Medlar leaves, and many other medicinal plants in treating leishmaniasis. These studies showed that herbaceous plant extracts have an inhibitory effect on the growth of parasites in some of them. The inhibitory effect of ginger extract on many agents in recent years has been reported [30-32]. Saki and et al. investigated the in vitro effects of *Zingiber officinale* on promastigotes and amastigotes of *Leishmania major* and *Leishmania tropica* [33]. The results showed that the hydroalcoholic extract of *Zingiber officinale* inhibited the growth of *Leishmania major* and *Leishmania tropica* promastigotes 24, 48, and 72 hours after in vitro incubation. The IC_{50} of hydroalcoholic extract of *Zingiber officinale* was 56 µg mL^{-1} for *Leishmania major* and 275 µg mL^{-1} for *Leishmania tropica* promastigotes after 72 hours. The IC_{50} of hydroalcoholic extract of *Zingiber officinale* was 75 µg mL^{-1} for *Leishmania major* and 325 µg mL^{-1} for *Leishmania tropica* amastigotes after 72 hours [33]. This study proved that the hydroalcoholic extract of *Zingiber officinale* has cytotoxicity properties, and *Leishmania major* has a lower resistance to the hydroalcoholic extract of *Zingiber officinale* than *Leishmania tropica* [33]. Duarte et al. studied the effect of *Zingiber officinale* extract and F10 fraction on promastigotes of *L. Amazonasis*. They observed the IC50 values of 125.5 µg mL^{-1} for the aqueous extract of *Zingiber officinale* and 49.8 µg mL^{-1} for the F10 fraction of *Zingiber officinale* F10 [34]. By Elamin resaech, the results showed that Curcumin had a potent antileishmanial effect, representing cytotoxicity against *L. major* promastigotes. At 80M, the survival in Curcumin treated promastigotes reached 22%; however, the median lethal concentration of Curcumin (LC_{50}) was 35M [35]. Kumar et al. investigated different extracts of *Curcuma longa* rhizome against *Leishmania donovani.* Their results showed that the methanolic extract had the maximum antileishmanial activity followed by chloroform and acetone extract against both promastigotes and amastigotes [36]. The present study showed that the survival rate of *Leishmania* promastigotes treated with hydroalcoholic extract of Ginger at 3 time points (24, 48, and 72 hours) was lower than Glucantime and Turmeric extract. The survival rate of promastigotes treated with Turmeric extract was similar to those treated with Glucantime but lower than those treated with a combined extract of Ginger and Turmeric at a concentration of 500 µg mL^{-1}. Oleanolic acid is a plant compound with anti-*Leishmania* properties derived from the marigold plant (*Calendua officinalis)*. This compound exhibits its leishmanicidal effect by inducing apoptosis. In a study by Ghosh et al., oleanolic acid was loaded on PLGA nanoparticles, and its efficacy in treating mice infected with *Leishmania donovani* was evaluated. The researchers indicated that the Nanocomposite reduced the parasitic burden by 78% ($P < 0.05$) in the spleen. In comparison, free oleanolic acid could reduce the parasitic load in the spleen by 67% [37]. Rajesh et al. observed the chemical and medicinal properties of Zingiberaceae. They indicated many active compounds in medicinal ginger, including flavonoids such as kaimferrols and gingerols [38]. Ginger has many usual pharmaceutical applications. It includes many biologically active chemical groups and various minerals and rare elements. The most crucial medicinal characteristic of Ginger extract that have been investigated in previous studies include antimicrobial, antifungal, anti-inflammatory, anti-parasitic and cytotoxicity, anti-diabetic, anti-cancer, and anti-oxidant effects [39]. In addition, the synthesis of different nano adsorbents such as activated carbon (AC) and carbon nanotubes (CNTs) exhibit inhibition properties against various bacteria similar to Turmeric extract and aroma oil of *Curcuma longa*. Also, it can be used to remove heavy metals and VOC pollution in various matrixes [40].

4. Conclusion

Since the hydroalcoholic extracts of ginger and *Curcuma longa* are safe and have not been found to have any toxic effects even at high doses, they can be used as suitable supplements to the treatment regimen of leishmaniasis and management of *Leishmania* parasites. The current study raised the anti-Leishmania and protective effects of the two plant extracts with increased concentration. With these properties, the hydroalcoholic extracts of ginger and turmeric can be combined with other compounds, such as nano-compounds, to produce effective herbal medicines against the retention time of *Leishmania* parasites. In our study, the IC50 values of ginger and turmeric plant extracts were similar to that of Glucantime, indicating that these extracts can be exploited as potential drug candidates for cutaneous leishmaniasis. We used the gas chromatography-mass spectrometry (GC-MS) method to analyze plant extracts to investigate plant extracts' effect on Leishmania promastigotes. This method showed that Ginger extract kills more parasites in the culture medium among the plant extracts than Turmeric extract and Glucantim.

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6. Conflicts of interest, Ethical Approval, and Funding/Support

The authors declare that they have no Conflict of interest. This study has an ethics committee license (IR.KMU.REC.1398.282). This study was financially supported (numbering project: 98000288) by the Kerman Medical University Research Council.

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