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Biological Activities of Iraqi Fig (*Ficuscarica*) CrudeEthanolic and Total Flavonoids Extracts

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Abstract

The present study focuses on the biological strategy for treating cancer and parasitic infections, such as leishmaniasis antiparasitic activity, forthe crude alcoholic extract of*Ficus* leaves and their extracted total flavonoids with a comparison between their effects.The flavonoids were extracted from the leaves of the mature *Ficuscarica*using the reflux extraction method. Total flavonoids were detected qualitatively by TLC techniquewhich demonstratedthat the plant was rich indifferent flavonoids, especially Rutin, Quercetin, Kaempferol, luteolin and others. Quantitatively, the plant total flavonoids content was 337.3 mg / 100 g fig leaves calculated as rutin.The biologicaleffects of the crude and purified total flavonoid on cell lines (L-20B and MCF7) and two parasites (*Leishmaniatropica* ,*Leishmaniadonvani)* were investigated. Maximum growthinhibition rates forthe total flavonoids onthe cell linesL20B andMCF7 reached 43 % at the concentration of 0.169 mg/ml and 28% at the concentration of 2.7 mg/ml, respectively, in comparison with the negative control. The ethanoliccrude extract had a low effect on L20-B cell line, while the inhibition rate forMCF-7 cell linereached 34% at a concentration of 0.084 mg/ml. For *Leishmaniatropica,*the total flavonoid and crude plant extractcaused maximum inhibition rates of 48% and 56%, respectively,at a concentration of 2.7 mg/ml for both. Cytotoxicity valueon *Leshmaniadonovani* was 20% for the crude extract at 1.35 mg/ml concentration, whereas it was11% for the total flavonoids at a concentration of 0.169 mg/ml. In conclusion, the differences in anticancer and anti-parasitic activitiesareattributed to differentcompounds present in each extract.

Keywords; *Ficuscarica*s leaves, L20-B cell line, MCF-7cell line, *Leishmania tropica*, *Leishmania donovini*.

الفعالية الحيوية لمستخلص الكحولي الخام ومستخلص الف لافونويدات الكليه لنبات التين العراقي

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الخلاصه

اكدت الدراسة الحالية على الاستراتيجية الحيوية لمعالجة السرطان والاصابات الطفيلية بالاخص الفعالية المضادة لنمو طفيلي اللشمانيا باستخدام المستخلص الكحولي الخام لاوراق نبات التين علاوة على مستخلص الفلافونويدات الكلية للنبات وعمل مقارنة بين تأثيريهما. حيث تم استخلاص الفلافونويدات الكلية لاوراق نبات

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التين الناضج بطريقة التبخير والتكثيف الانعكاسي. وتم تقييم الفلانويدات الكلية نوعيا بطريقة كروماتوغرافي الطبقة الرقيقة حيث اتضح ان النبات غني بعدة انواع من الفلافونويدات بضمنها الروتين والكوارستين والكامفيرول وايضا اللوتيولين ومركبات فلافونويدية اخرى لم يتم التعرف عليها. اما كميا فالنبات يحتوي على فلافونويدات كلية تقدر بـ337,3 ملغم/100 غم اوراق تين احتسبت على اساس الفلافونويد القياسي الروتين. اما التأثير البايولوجي لكلا المستخلصين الكحولي الخام والفلافونويدات الكلية على خطين خلويين (L20B and MCF7)وعلى نوعين من طفيلي اللشمانيا(*L. tropica L. donvani*) فقد تم التطرق لها في هذا البحث. فقد وجد ان اقصى تثبيط للفلافونويات الكلية في نمو الخطين الخلويين L20B and MCF7 قد بلغ 43% عند التركيز 0,169 ملغم/مل للخط السرطاانى الاول وبقيمة 28% عند تركيز الفلافونويدات 2,7 ملغم/مل بالنسبة للخط الخلوي الثاني. في حين ان المسخلص الكحولي الخام لم يكن ملحوظا تجاه الخط الخلويL20B بينما بلغ اقصى تثبيط لخلايا MCF7 ما قيمته 34 % عند تركيز 0,084 ملغم/مل. اما فعالية المستخلص الفلافونويدات الكلية والمستخلص الخام على نمو طفيلي اللشمانيا الجلدية فقد بلغ اقصى تثبيط 48% و56% عند التركيز 2,7 ملغم /مل على التوالي. اما السمية الخلوية تجاه الطفيلي *Leshmania donovani* فبلغت اقصاها للمستخلص الخام 20% بتركيز 1,35 ملغم/مل وان الفلافونويدات الكلية سببت بتثبيط اقصاه 11% عند التركيز 0,169 ملغم/مل.

Introduction

Cancer is the second cause of death in the world. In 2015, it was responsible for 8.8 million deaths. Genetic influences have been long suspected[1].

Parasitic diseases such as malaria, leishmaniasis, and trypanosomiasis represent a significant global burden and pose a great challenge to drug discovery and delivery, due to their intracellular nature and disseminated locations. Moreover, the poor rate of discovery in the anti-parasitic segment seen in last few decades has necessitated effective management of the existing drugs by modulating their delivery[2].

Medicinal plants are gaining importance in these fields of research. *Ficus carica* (Fig),family Moraceae, is one of the medicinal plants used in different illness conditions. Ithas been cultivated from the Middle East and West Asia and spread to many regions in the world. Products of *F. carica* are widely used as food sources and medicine to treat various diseases[3]. The plant is rich in minerals such ascopper, mangnesium, potassium, calcium, and vitamin K. It is also a good sourceof flavonoids and polyphenols, including galic acid , chloragenic acid, syringic acid, catechin, picatechin and rutin [4].Fig fruits are astringent to bowels, tonic, and useful in the treatment of leucorrhoea, blood discharges, leprosy, menorrhagia, and intestinal worms.While leaves have an astringent usein urinary disorders and bronchitis. Bark is useful in asthma and piles, whereas latex is applied externally on chronic infected wounds to alleviate edema andpain and to promote the healing[5].

Materials and Methods

1.Plant collection and classification

The samples of Fig leaves were collected from the Al Hawejaarea/ Kirkuk-Iraq and classified in the Faculty of Agriculture, Baghdad University. The samples were taken to the laboratory after cleaning well from suspended soil. They were dried at room temperature (25^{o}C) and manually grinded, then packaged in sterile containers and kept away from direct light until use.

2.Samples preparation

2.1.Preparation of leaves crudeethanolic extract

About 50 g dried powdered fig leaves were macerated in 400ml of 70%ethanol for 3 days at room temperature then filtered. The filtrate was dried by rotary evaporate at 45C^{0} till dryness. The residue from the crude extract was weighted and kept in a dark container for further chemical and biological assays[6].

2.2. Extraction of the total flavonoids from fig leaves

About 30 g of powdered plant samples were placed in a 500 ml glass flask and then 300 ml distilled water with10% v/ v HCl was added. Reflux extraction was performed for 8 hours continuously to ensure that the cleavage andbreakof the glycoside linkage betweenthe flavonoids and the aglycon part was obtained. The plant extract was filtered and cooled. The aglycan portion that

possesses biologic effects was extracted by ethyl acetate in a portion of 1:1. The extraction was repeated three times using a separating funnel. The acetate layer was combined and washed with distilled water to remove HCl residues. Finally, the acetate layer was dried with a rotaryevaporator at 45 ° C. The residue was weighted and kept for further analysis [7].

2.3- Preparation of stock solution

0.4 g of the crudeethanolic residue and total flavonoids residue were re-dissolved, each in 75 ml distilled water to obtain a final concentration of 5.33 mg/ml.

3. Phytochemical tests for the crude extract

For the purpose of these tests, about 10 ml of the crude extract solution with a concentration of 5.33 mg/ml was used to identify the leaves chemical components,as follows [8]:

A. Detection of Tannins

Few drops of 1% lead acetatesolutionwere added to the plant extract. A white precipitateindicated the presence of tannins.

B. Detection of reduced sugar

An aliquot of 1 ml plant extract was mixed with 2 ml of Benedict reagent. The mixture was placed in a boiling bath for 5 minutes and left to cool. The red deposit indicated the presence of reduced sugar groups.

C. Detection of alkaloids

According to Dragangroff test, about 60mg of bismuth subnitrate was dissolved in 0.2ml HCL to obtainsolution A. Solution B contained 600mg potassium iodide in 1 ml distilledwater. The combined solution [A + B]was added to the plant extract, forming anorange-brown precipitatewhich indicatedthe presence of alkaloids.

D. Detection of the saponins

Formation of a foam at the top whenshaking the plant extractindicated the presence of saponins.

E. Detection of Flavonoids

Based on the alkalinereagent test, sodium hydroxide 2N solution was mixed with a few amount ofplant extract solution and left for few minutes. The yellow colorobtained indicated the presence of flavonoids.

F. Detection of Polyphenolic compounds

Addingferric chloride 1% solution to the plant extract solution caused the formation of a green-brown deposition, which is an indication of polyphenolic compounds.

4. Determination of Fig leaves' Total Flavonoids

4.1. Qualitative assay

Standard flavonoids, namely rutin, quercetin, kaempferoland luteolin, were prepared in ethanol.Thin layer chromatography (TLC) was carried out using a silica coated 60 plate with a thickness of 0.1 mm,while the mobile phase containedtoluene : ethyl acetate: formic acid in a ratio of 36: 12: 5.

The types of flavonoids separated couldbe detected in corresponding to standard flavonoid spots,whereas the R_f valuewas calculated as in the following equation:

$$\text{Rf value} = \frac{\text{Distance traveled by each flavonoid}}{\text{Distance traveled by the mobile phase}}$$

Each flavonoid could be detected separately after exposure of the silica plate to the UV light at 254 nm wave length[9].

4.2. Quantitative Assay

Rutin standard flavonoid solutions (0.3125, 0.15625, 0.625, 1.25 mg/ml) were prepared in ethanol. About 1 ml of stock flavonoids extract solution (5.33 mg/ml)was transferred to a glass tube, and of 1 ml rutin standard solution from each concentration wasplaced in separated glass tube, then1 ml of 5% sodium nitrite solution was added to all tubes, mixed and left atroom temperature for 5 minutes. To each tube, 2 ml of aluminum chloride10% was added, mixed and left for another 5 minutes at room temperature. Finally, 5 mL of 1N NaOH solution was added and the resulting color was read with spectrophotometer at 510nm wavelength. A standard curve was generated based on the absorbance of each standard solution againsteach concentration. Thestraight line equation was detected to calculatethe total amount of flavonoids in the extracted plant[10].

4.3. Anticancer activity*in vitro*

In this study,thegeneral protocol reported by Chliet*et,al.* and Fresheny[11,12]wasappliedagainst two types of cancerous cell; the L20B cells and MCF-7breast cancer cells. Briefly, different concentrations o (0.084, 0.169, 0.338, 0.675, 1.35 and 2.7 mg/ml) of the crudeethanolic extract and total flavonoids solution were prepared in a medium. Two plateswere separately seeded with 100µl/ well of one kind of cancer cells suspended in growth medium and incubated for 24 hours for monolayer confluent. An aliquot of 100 µl from each plant extract concentration was added to a number of cultured wells in duplicate manner and incubated after all additions at 37^0C overnight for 24 hours. A volume of 10 µl of the freshly preparedMTT coloring reagent (5 mg/ml) was added to all wells. The plate was incubated at 37^0 C for at least three hours. Finally, about 50 µl of DMSO was added to all wells and incubated for 10 min. The control was indicated as cell culture suspended in medium without plant extract. The absorbance of the treated and untreated wells was measured at 620 nm with an ELISA reader. The growth inhibition ratio was calculated as follows:

Growth Rate inhibition % = $\frac{Control-Treatedcell}{Control}\times 100$ [11]

4.4. Anti Leishmanial activity *in vitro*

The anti-leishmanial activity of *Ficuscaricas* extracted flavonoids and the crudeethanolic leave extract was studied against two species of *Leishmanial* parasite in the promastigote form; *L. Tropica*and*L.donovani*. A colorimetric method described by Mahmoud *et al.*[13] was applied. Initially, about 100 µl from both species of *Leishmania*promastigoteswas suspended in all of the 96 wells of a tissue culture plate in a concentration of10^6 parasites/ ml. Then, 100 µl/ of various concentrations (0.084, 0.169, 0.338, 0.675, 1.35 and 2.7mg/ml) ofeach extract solution were prepared in RPMI medium. Aliquots of 100 µl of each prepared treatment solution at different concentrations were added to the two seeded well plates and incubated at 26 ^{0}C for 24 hours. About 10 µl of the freshly prepared solutionof theMTT coloring reagent () (5 mg/ml) was added to all wells. The plate was incubated at 26 ^{0} C for at least three hours. Finally, about 50 µl of DMSO was added to all wells and incubated for another 10 min. The control was indicated as promastigotes cultured in complete medium without plant extract. The absorbance of treated and untreated wells wasmeasured at 620 nm with anELISA reader. The growth inhibition ratio was calculated as follows:

Growth inhibition Rate % = $\frac{Control-Treatedcell}{Control}\times 100$ [13]

Results and Discussion

1. Plant Classification:

The plant was classified at the Faculty of Agriculture, Baghdad University, as *Ficus carica.*

2.PlantExtract Yields

The crudeethanolic extract of 50 g of the dried *Ficuscrica* leaves yielded about 8.5 gresidue, while the total flavonoids extracted from 30 g of thedried plant leaves yielded about 0.42 g residue.

3. Phytochemical tests for the crude extract:

Table-1 indicates the main active groups that present in the crude alcoholic extract of plant leaves.

Table 1-The main active groups in the crude alcoholic extract of fig leaves

TEST	RESULT	COMMENTS
. Tannins	++	Whiteprecipitate
Reduced sugar	+++	Heavy Red precipitate
Alkaloids (Dragangroff)	+	little brown precipitate
Saponines	++	Foam formation
Flavonoids	+++	Bright yellow color
Polyphenolic compounds	+++	Brown precipitate

4. Determination of Fig leaves' Total Flavonoids

4.1. Qualitative Assay

Flavonoids were extracted from the plant and were identified by thin layer chromatography (TLC). Spots were obtained by UV exposure ofthe plate to a 254nm wavelength, in comparison with standard flavonoids(Figure-1).

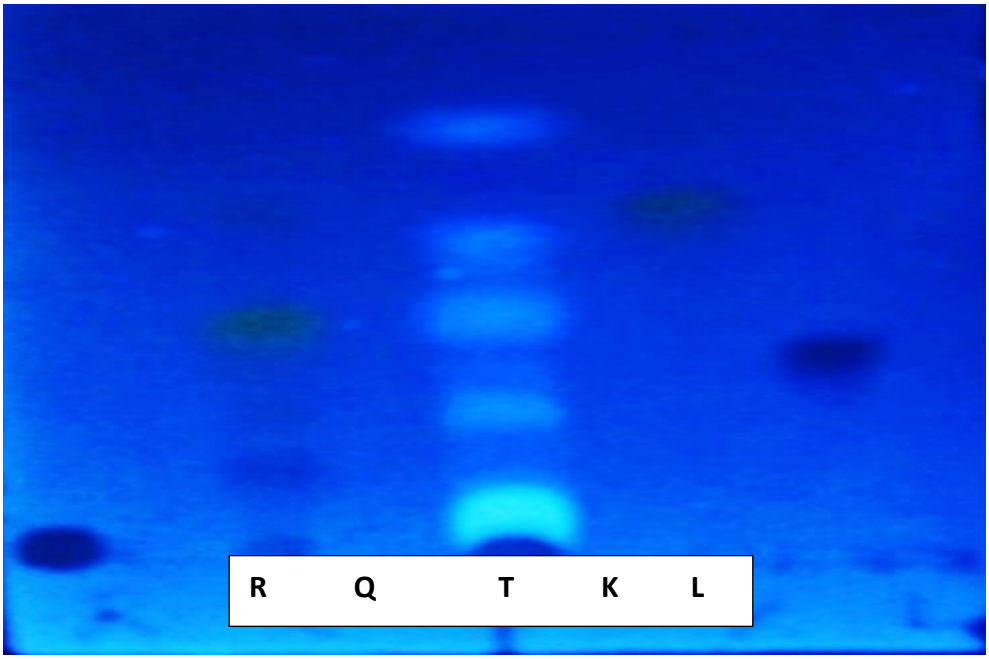

Figure1-TLC chromatogram of the extracted total flavonoids(F) corresponding to standard flavonoids; Rutin(R), Quercetin(Q), Luteolin(L), Kaempferol(K).

As shown in Figure-1, the total flavonoids extracted from the plant leaves containeddifferent types, namely quercetin, kaempferol, luteolin and others, the levels of whichwere detected by the calculation of R_F values shown in Table-2.

Table 2-Thevalues of Rf for different flavonoids and the extracted flavonoids.

Flavonoid Type	Rutin	Quercetin	Luteolin	Kaempferol	Extracted flavonoids
R_F value	Base line	0.28	0.2	0.53	Base line, 0.2, 0.28, 0.53 and 0.9

4.2.Quantitative Assay

The amount of flavonoids found in *F. caricas* leaves was estimated using the standard rutin curve.Table-3 shows the absorption values of the standard flavonoid at different concentrations as well as ofthe extracted total flavonoids ,where the straight line equation was obtained as shown in Figure-2.

Table 3- Absorption values of the standard flavonoids (rutin)at different concentrations and the extracted total flavonoids of plant extract.

Absorption at (510 nm)	**Rutin standard solution(mg/ml)**
0.135	0.15625
0.341	0.3125
0.602	0.625
1.003	1.25
2.750	2.5
1.144	Total flavonoids of the plant extract

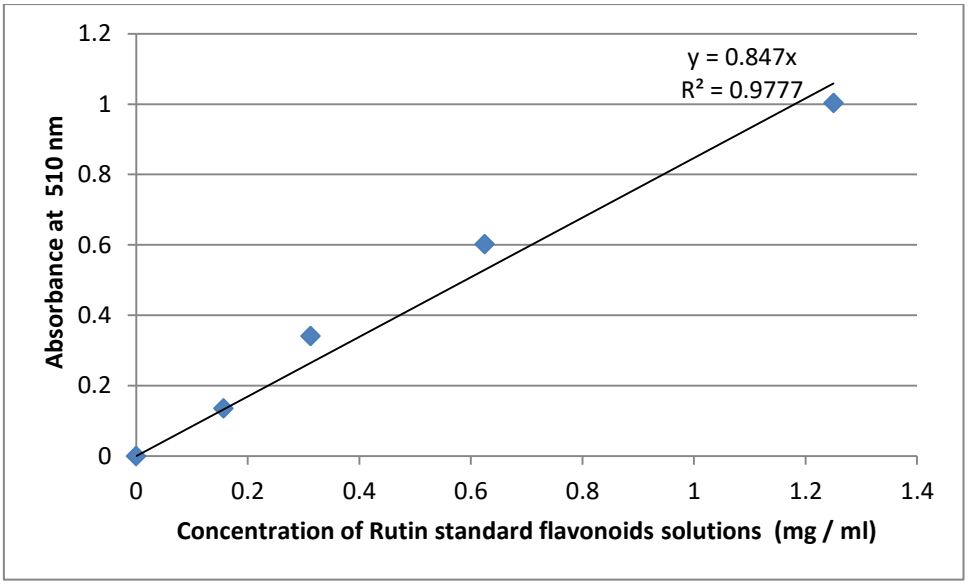

Figure 2-Rutin standard curve From the equation of the straight line, the concentration of the total flavonoids inthe extract was calculated as follows:

Y = 0.847X

X = Y / 0.847

X =1.144/ 0.847

X=1.35mg/ml total flavonoids in each 5.33mg residue that dissolved in 75 ml yielded from the extraction of 30 g plant Total flavonoids / 100 g *ficus caricas* dried leaves= 1.35mg/ml X 75 ml X 100 g / 30 g= 337.5 mg.

4.4. Anti-Cancer Activity on L20-B Cell line

As shown in Figure-3, different cytotoxic effects were obvious for both *F.carica* leaves ethanolic crude extract(Red) and the total flavonoids(Green) on L20-B cell line.

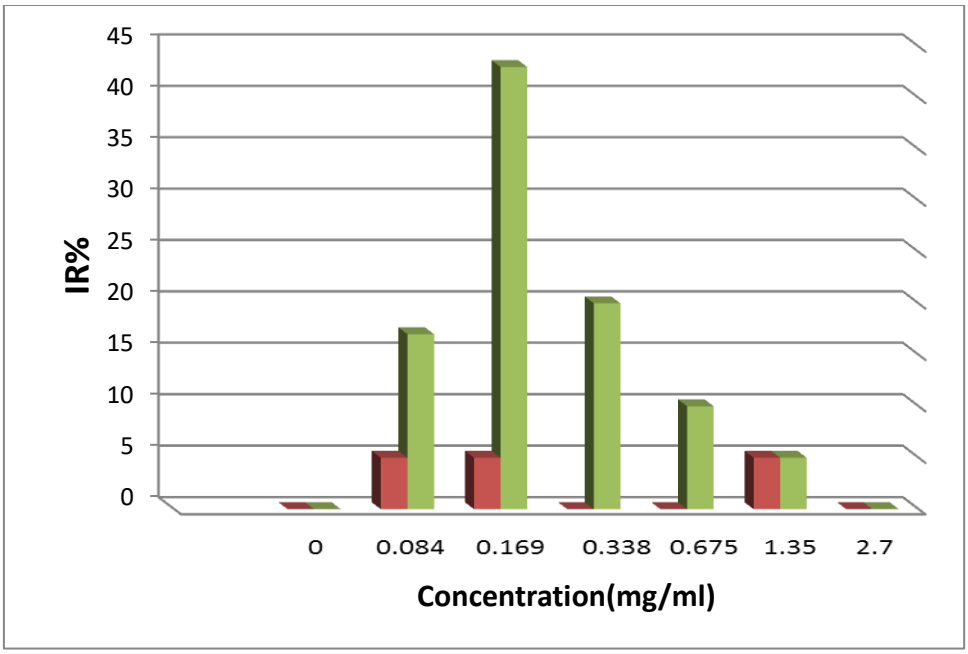

Figure 3-Cytotoxic effect for both crudeethanolic fig leaves extract(Red) and the total flavonoids(Green) on L20-B cell line

As shown in Figure-3, the extracted total flavonoids showed a more potent effect on the cell growth than the crude, .Each of the individual flavonoids extracted in the present study possess important roles in controlling and treating different kinds of cancers in the three stages, as flavonoids act as free radical scavengersand potent antioxidants [14]. The growth inhibition rate for L20-B cancerous cell line was constant in almost all plant crudeconcentration*. While in the case of *F.carica* total flavonoids, the cytotoxic effect against this cell line appeared even in small concentrations, reaching a maximum inhibition rate (43%) at a total flavonoid concentration of 0.169 mg/ml.

These effects could be mediated through phenolic acids, chlorogenic acids, flavones, and flavonols that are present in *F.carica*.[15]. Quercetin compounds are the main phenolic compounds found in *F.carica*. Quercetin has the ability to stimulate the apoptosis of many cancer cells by stimulating the release of cytochrome c from the mitochondria[16]. *F. carica* also contains fibers, vitamin A, vitamin C, calcium, magnesium, and potassium which are needed by the body. Other bioactive compounds of *F.carica* are arabinose, β-amirin, β-carotene, glycosides, β-sitosterol, and xanthol, which are antioxidant compounds [17,18].

F.carica has previously been demonstrated to inhibit the growth of HeLa cancer cells and MDA-megabyte (MB)-231 breast cancer cells [19].

4.5. Anti-Cancer Activity on MCF-7 Cell line

Figure-4 shows the different effects for both plant extract on the breast cancer cell line MCF-7, as indicated by the values of inhibition rate.

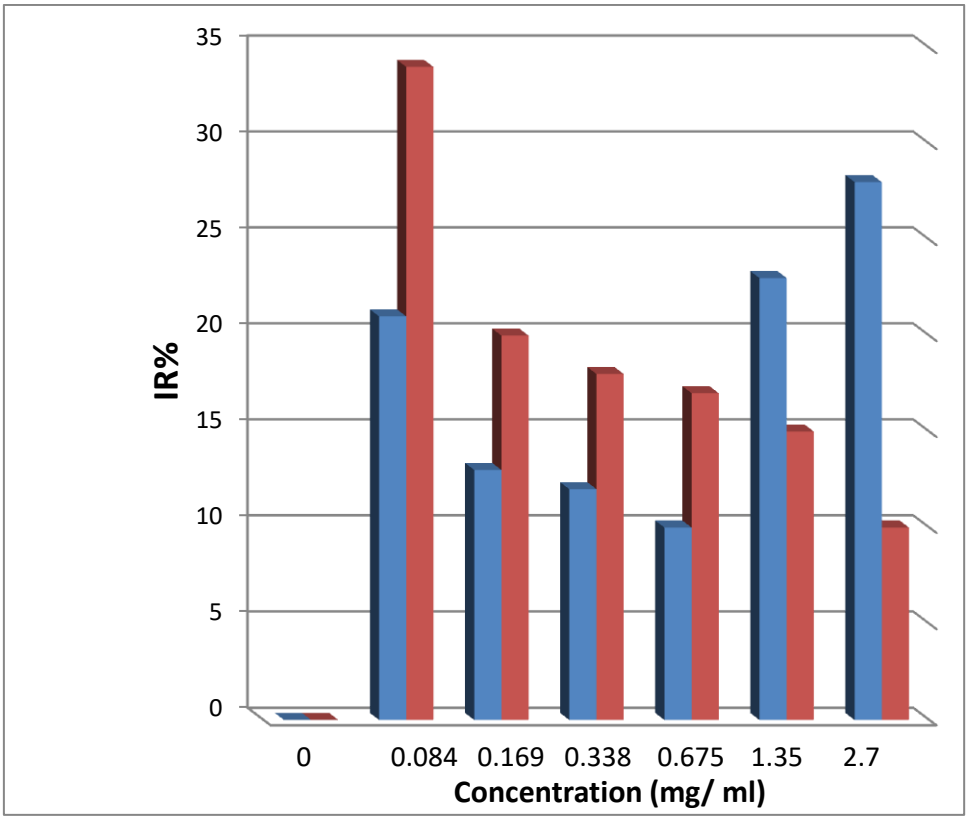

Figure 4-Cytotoxic effect for both crudeethanolic*F.caricas*leaves extract(Red) and the total flavonoids(blue) on MCF-7 cell line.

Both types of extracts affected the growth of the breast cancer cell line MCF-7 in a different manner. As shown in Figure-4, the crudeethanolic extract(Red) inhibited the cell growth in all concentrations, where the most potent effect appeared at the lower concentration(0.084mg/ml) which caused a maximum inhibition rate of 34%. Total flavonoids extracted from fig leaves resulted in MCF-7 growth inhibition(blue) from 0.084 mg/ml which give rate inhibition 20% up to 28% at concentration 2.7mg/ml.

One study published in 2018[20] indicated that breast cancer has substantially higher incidence than any other cancer diseases in women,and are categorized into three basic groups: human epidermal growth factor receptor-2 (HER2/ERBB2), estrogen receptor (ER) positive, and triple-negative breast

cancer (TNBC, also known as basal-like breast cancer)[13]. It was found that there are some key molecules in breast cancer that are tightly involved in proliferation or apoptosis of breast cancer cells. These include *GATA3, p53, Bax, p21, ELF5*, and cyclin-dependent kinases *(CDKs)*, which can affect the viability of cancer cells by repairing damaged DNA, influencing the cell cycle, or inducing apoptosis[21- 23].The main cause of death in breast cancer is metastasis, and many molecules are involved in the process, including *MPP2, TIMP1*, and *TIMP2* [24- 26].

Another study investigated the molecular mechanisms of the effects by analyzing the expression of key breast cancer biomarkers which are crucial to cell proliferation andcell cycle.Migration of the extracts from *F. carica* leaves indicated that the leave components have anticancer effects on triple-negative breast cancer MDA-MB-231 cells (TNBC) cell line, which was the most difficult subtype of breast cancer to treat. The reports also suggested that *F. carica* leaves might be a good source to develop drugs for suppressing cancer-cell growth and migration and to treat TNB cancer [27].

4.6. Antiparasitic Activity against*Leishmania tropica*

Figure-5 shows the*in vitro* effects of the *F.carica*crude extract(blue)at different concentrations and the total flavonoids (Red) against *Leishmaniatropica*.

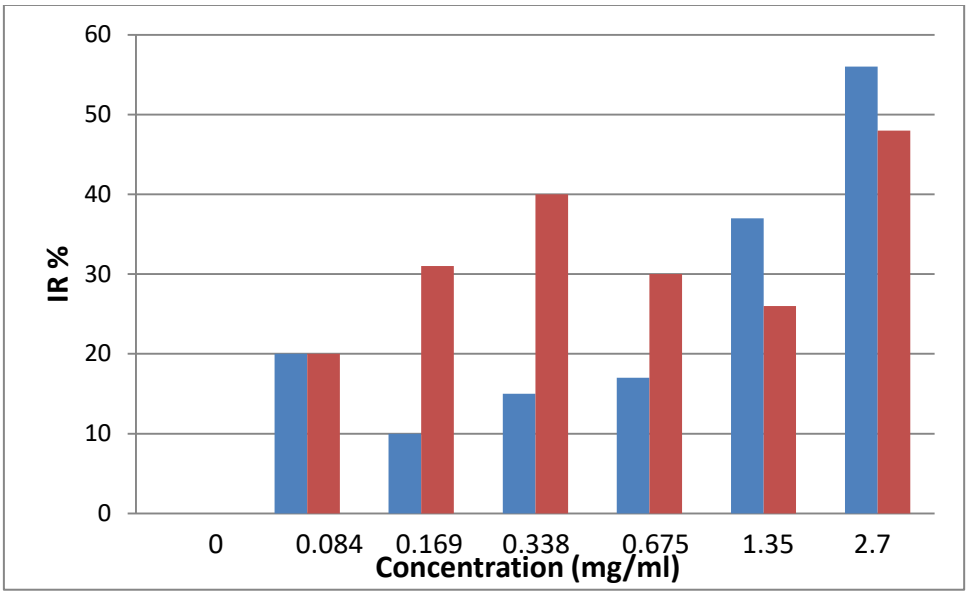

Figure 5-Anti-parasitic effectsof different concentrations of the *Ficuscarica*CrudeEthanolic Extract(blue) and Total Flavonoids (Red) against *Leishmaniatropica in vitro*.

Both Figures leaves extracts affected the growth of *leishmania tropica* at all concentrations and in a dose dependent manner. The maximum cytotoxic effect appeared at the concentration of 2.7 mg/ml for both the crude and total flavonoids extract, reaching 56% and 48%, respectively.

There are only fewstudies about antileshmaniasis activity for *Ficuscarica*different plant parts. A review [28]noted that the milky sap and ethanolic extract for two species,*Ficuscarica* and *Ficus religiosa*,had antiparasitic effectsagainst*Aedesaegypti* and *Pheretima posthuma* parasites, respectively.

Recently, the number of researches on antileishmanial agents significantly increased for two reasons. Firstly,several treatments such as antimony derivatives remain toxic and expensive.Secondly,several*Leishmania* species showed the resistance against synthetic molecules, and therefore the emergence and reemergence of infectious diseases. These two situations have oriented pharmacological researches on antileishmanial drugs to screen plants components that possess a selective efficacy and tolerable safety. Medicinal plant secondary metabolites such as volatile oil, flavonoids, polysaccharides, alkaloids and others, showed several pharmacological properties including antibacterial, antioxidant, and anticancer ones, which enhanced the researches to project them as antiparasitic compounds [28].

4.7. Antiparasitic Activity against *Leishminia donovani*

The effectsof *Ficuscarica*crudeextract and total flavonoids on the growth of *Leishminiadonovani*is shown in Figure-6.

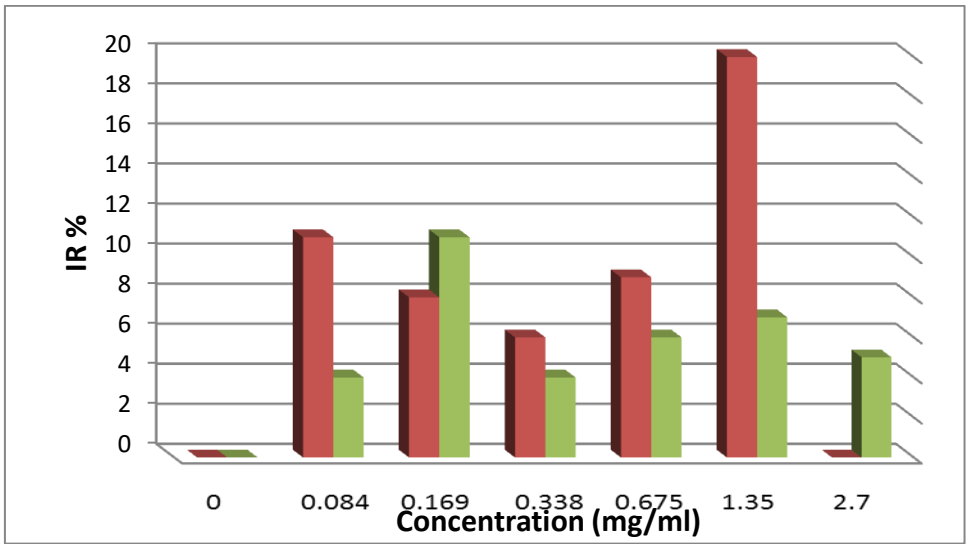

Figure 6-Anti-Parasitic Effect of the *Ficuscarica*crudeethanolicextract(Red) and total flavonoids (Green) against *Leishmania donovani in vitro*at different concentrations.

Crudeethanolic extract for *Ficus caricas* leaves(Red) possessed potent antileihminiasis effects in all concentration,giving a maximum inhibition rate(20%) at the concentration of 1.35mg/ml. While, Fig total flavonoids showed potent cytotoxic effect (11%) in a low concentration (0.169 mg/ml).
The differences in the biological activity for both crude and total flavonoids of *Ficus* plant were due to the differencesin the active constituents present in each one. Studies reported that the major components in the fig leaves ethanolic extract were thefuranocoumarins including psoralen and bergapten[29]in addition totriterpenes such as lupeol acetate[30]. Other valuable ingredients are phenols, anthocyanins, fructose, glucose, and sucrose were identified from the figs [31], while anotherfinding reported that the fruit has phyto-sterols [32].

Conclusions

The differences in anticancer and anti-parasitic activitieswere attributed to differing compounds present in each extract.

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