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# The Effects of *Tropaeolum majus* and *Cupressus lusitanica* on the Genus *Paramecium* Protozoa

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Author WN designed the study and wrote the draft manuscript. Author BN wrote protocol and carried out laboratory experiments under author WN supervision. Authors JN and AN contributed in commenting and facilitating necessary materials for the research work and manuscript preparation. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** This work was undertaken to evaluate the impact of two different medicinal plants known as *Tropaeolum majus* and *Cupressus lusitanica* on protozoa, genus *Paramecium*.
**Methodology:** For the culture of *Paramecia*, the method called "The growth of *Paramecium* in pure culture of bacteria" was employed by using an infusion of dried grasses and some nutrients. The aforementioned nutrients were food for bacteria and the bacteria were food for *Paramecia*. Microscopic examination allowed seeing if they have really grown, if they are active or constituted by all organelles including two nuclei: Macronucleus and Micronucleus. For the preparation of plant extracts, three types of plant extracts have been prepared: Boiled extracts, Crude extracts and

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Solvent extracts. Serial dilution was used for dissolution of extracts.
**Results:** Different concentrations of the plants extracts were applied on the *Paramecia* sample. After 24 hours of incubation period, microscopic observation was done to test the antimicrobial effects. For both plants, the crude and the solvent extracts showed anti-protozoal effects at concentrations 1 and $10^{-1}$ just after 24 hours. The boiled extracts and the low concentrations ($10^{-2}$ & $10^{-3}$) of crude and solvent extracts were showed anti-protozoal effects, but after 5 days long incubation period. These effects were represented by: the death of *paramecia*, their inactivation, and disappearance of some organelles, cilia and/or one nucleus.
**Conclusion:** From this study we conclude that *Tropaeolum majus* and *Cupressus lusitanica* can be used as anti-protozoal medication in order to treat protozoan's infection mainly Trichomoniasis. However, to improve its potency, further study is recommended on the isolation and purification of the active ingredient components.

*Keywords: Anti-protozoal activity; Paramecium; Tropaeolum majus; Cupressus lusitanica.*

## 1. INTRODUCTION

*Paramecium*, a genus of unicellular ciliated protozoa, is commonly studied as a representative of the ciliate group. Because some of the species are readily cultivated and easily induced to conjugate and divide, they have been widely used in classrooms and laboratories to study biological processes. They are found everywhere in nature such as ponds, swamps and in dried grasses. They are not harmful to human and other animals or to plants [1]. *Tropaeolum majus* (garden nasturtium, Indian cress or monks' cress) is a flowering plant in the family *Tropaeolaceae* [2]. It has long been used in herbal medicine as a disinfectant and wound-healing herb, and as an expectorant to relieve chest conditions [3]. *Cupressus lusitanica*, growing up to 40 m tall, is an evergreen conifer tree with a conic to ovoid-conic crown [4]. It is widely cultivated, both as an ornamental tree and for timber production [5]. It is also used for its antifungal properties [6].

Plants are rich in a wide variety of secondary metabolites such as essential oils, tannins, terpenoids, alkaloids, flavonoids glycosides etc, which have been found to have antimicrobial properties [6,7]. For example the essential oils from leaves of *C. lusitanica* have been studied for its antimicrobial activity against fungal and bacterial agents [7]. Plants have been used as traditional medicine since time immemorial to control bacterial, protozoal, viral, and fungal diseases [8]. Generally, drug plants are unique for containing compounds that are end-products of long biosynthetic pathways and are usually not needed in such plants' metabolic processes [9]. These compounds, called secondary metabolites, are usually produced in different parts of the plants like the roots, leaves, fruits and seeds and then translocated to other parts of the plants for storage, and they are of great importance in controlling infectious disease commonly found in the world including protozoal infections [9,10].

The determinations of potential anti-protozoal effects for plant extracts may provide information for further use in medicinal practice. The aim of the present study was to determine the anti-protozoal effects of *T. majus* and *C. lusitanica* plant extracts against non pathogenic microorganisms (*Paramecia*). The use of these medicinal plants as source for relief from illness of Trichomoniasis, an infection caused by protozoal parasite known as *Trichomonas vaginalis*, is common in Rwandan traditional medicine. Systematic screening of them may result in the discovery of novel effective compounds.

## 2 MATERIALS AND METHODS

### 2.1 Collection and Extraction of Plant Materials

The fresh leaves of *T. majus* and *C. lusitanica* were collected from local gardens and surrounding forest in Kigali city respectively. Leaves of the plants were washed with distilled sterile water and cut into small pieces and then air dried at room temperature. The dried plants were grounded by using an Electric blender and then sieved through a sieve of Muslin mesh and Whitman filter paper in order to get a very fine powder for long storage and easy transport.

Thereafter, extractions of both plants were done by using three different methods as follows: - Solvents extraction, Extraction by boiling and Crude extraction. Solvent extraction by methanol, chloroform and hexane for *T. majus;* and ethanol for *C. lusitanica* were made.

For the extraction of *T. majus*, 20g of the powdered leaves were soaked in a mixture of 20 ml of hexane ($C_6H_{14}$), 20 ml methanol ($CH_3OH$), and 20 ml of chloroform ($CHC_{l3}$) at ambient temperature. Solvent extraction was carried out for 24 hours under shaking condition at 150 rpm. The filtration of the extracts was then done by using filter paper. Solvents were removed under the vacuum at 45˚C using a Rotor Vapor. After this, each fraction was become evaporated on a Vacuum Rotary Evaporator, under reduced pressure, for removing organic solvent. Then extracts were kept in the freezer at 20℃.

For the extraction of *Cupressus lusitanica*, 60 g of the powdered plant was soaked in 240 ml of 80% ethanol at ambient temperature for 24 hours under shaking condition at 150 rpm.

The filtration of the extracts was then done by using filter paper. Solvents were removed under the vacuum at 45℃ using a Rotor Vapor.

Extraction by boiling was done with a heater, moist-heat 100 ml of distilled water in a beaker until it boils. Then 100 g of *T*. *majus* was added. After cooling of the solvent was made filtration was done by using Muslin mesh and Whitman filter paper.

Preparation of *T. majus* crude extract was made by grinding washed fresh leaves in artificial blender and then filtrated with a Muslin mesh and Whitman filter paper. The obtained fresh extract was kept in the freezer at 20℃. The same procedures of boiling and crude extractions were followed for *Cupressus lusitanica*.

## 2.2 Collection of *Paramecia*

*Paramecia* were collected by using an infusion of dried grasses, pond water and some nutrients (Sosoma flour and glucose). These nutrients were used as food for bacteria and the bacteria were fed on by *Paramecia*.

## 2.3 Culture of *Paramecia*

Culture medias for bacterial and *Paramecia* growth were prepared by adding a mixture of moist grasses, river or pond water and a small quantity of nutrients such as glucose and Sosoma flour powder (Supplied by SOSOMA Industries Ltd.) in a beaker. Small quantities of nutrients were added to prevent fermentation because the alcohol formation may create bad effects on the *Paramecia*. The beakers were kept at ambient temperature for incubation.

The growths of *Paramecia* were confirmed after each 24 hours of incubation. Specimens, aseptically taken from the medias, were examined microscopically to study the growth of *Paramecia* in the culture. Phase contrast or dark field at a lower magnification reveals cilia and organelles. Bright field is needed to distinguish colors of food vacuoles. Nuclei appear white, one smaller (Micronucleus) than the other (Macronucleus). The optimal growth period was found to be between 5 to 8 days of the 11 days of different growth phases.

## 2.4 Anti-protozoal Property Screening

Two panels of assessments were prepared. One was test panel and the other was control panel. For the test panel 4 test tubes were prepared by serial dilution for each plant's extract. Then in each test tube 3 ml of the specimen of *Paramecia* was added. After waiting for 24 hours of incubation period, changes were observed for the consecutive four days. As a control panel 2 test tubes were kept containing the original specimen.

After 24 hours, microscopic examinations were done to observe changes such as inhibition of their movement, loss of some organelles or cilia, inactivation or death; at each concentration, at different time and on six different plants' extracts. Phase contrast or dark field at a lower magnification also reveals cilia and organelles to see if they are again present. Lugol's Iodine stain was also used to visualize cilia, organelles, the micronucleus, and the macronucleus.

# 3. RESULTS

## 3.1 Results for *T. majus* Effects

Table 1 and Fig. 1 present the summary of anti-protozoal activeties of all the three *T. majus* extracts. These results showed that *Paramecia* were rapidly sensible to solvents and crude extracts within the first day incubation period at high concentrations (1, $10^{-1}$). They were also sensible to the low concentrations ($10^{-2}$, $10^{-3}$) of those extracts after a medium period of time (4 days) incubation. But for boiled extract, the sensitivity begins by weakening *Paramecia* until they die after a long period of time in this case after 5 days incubation.

## 3.2 Results for *C. lusitanica* Effects

Table 2 and Fig. 2 show the summary of anti-protozoal activities of all the three extracts of

*C. lusitanica*s. This anti-protozoal activity study showed that *Paramecia* were rapidly sensible to solvents and crude extracts at higher concentrations (1, $10^{-1}$) within one day of incubation. At the same time it was found to be sensible to the low concentrations ($10^{-2}$, $10^{-3}$) of those extracts after four days of incubation period of time. But for boiled extract, the sensitivity begins by weakening *Paramecia* until they die after 5 days of long period of incubation.

**Table 1. Anti-protozoal activity test of *T. majus* against to *Paramecia* (boiled, solvent and crude extracts)**

| Time in days | Concentrations | Number of *Paramecia* per microscopic field | | | | Appearance | Movement |
|---|---|---|---|---|---|---|---|
| | | Control | Boiled extract | Solvents extract | Crude extract | | |
| 1 (24hrs) | 1 | 200 | 50 | 0 | 0 | Weak | Moving slowly |
| | $10^{-1}$ | | 100 | 0 | 0 | Weak | Moving slowly |
| | $10^{-2}$ | | 200 | 10 | 10 | Normal | Moving faster |
| | $10^{-3}$ | | 500 | 50 | 50 | Normal | Moving faster |
| 4 | 1 | 200 | 20 | 0 | 0 | Weak | Moving slowly |
| | $10^{-1}$ | | 40 | 0 | 0 | Weak | Moving slowly |
| | $10^{-2}$ | | 80 | 5 | 5 | Weak | Moving slowly |
| | $10^{-3}$ | | 200 | 20 | 20 | Normal | Moving faster |
| 5 | 1 | 190 | 5 | 0 | 0 | Weak | Moving slowly |
| | $10^{-1}$ | | 10 | 0 | 0 | Weak | Moving slowly |
| | $10^{-2}$ | | 20 | 0 | 0 | Weak | Moving slowly |
| | $10^{-3}$ | | 40 | 2 | 2 | Weak | Moving slowly |
| 6 | 1 | 160 | 0 | 0 | 0 | Weak | Moving slowly |
| | $10^{-1}$ | | 5 | 0 | 0 | Weak | Moving slowly |
| | $10^{-2}$ | | 10 | 0 | 0 | Weak | Moving slowly |
| | $10^{-3}$ | | 20 | 0 | 0 | Weak | Moving slowly |

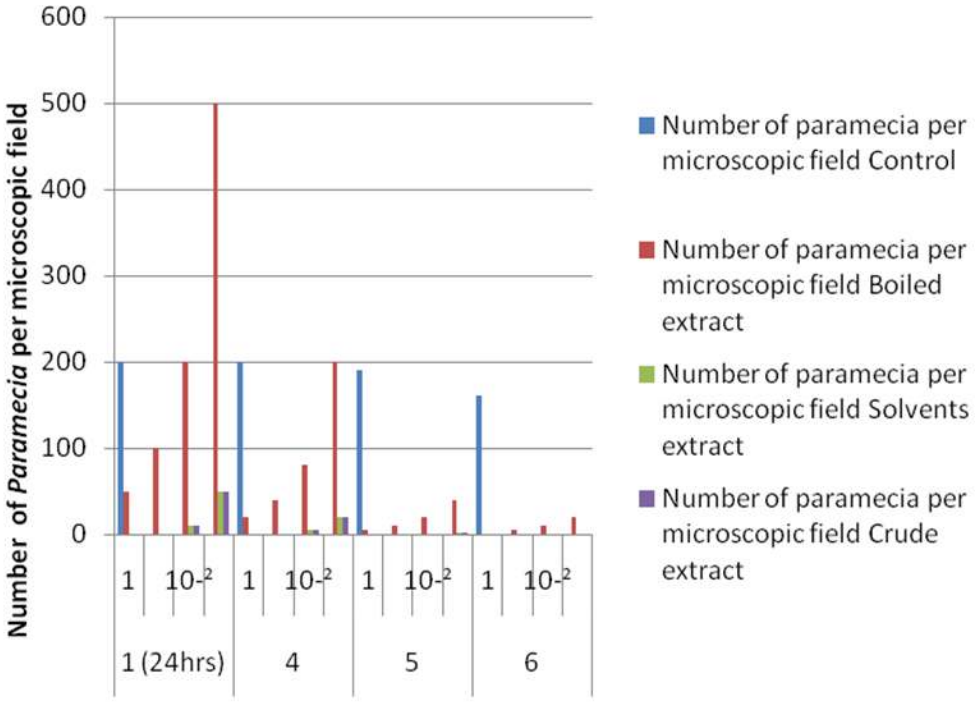


**Fig. 1. Anti-protozoal effects of *T. majus* different extracts on *Paramecia***

**Table 2. Anti-protozoal activity test of *C. lusitanica* (boiled, solvent and crude extracts)**

| Time in days | concentrations | Number of *Paramecia* per microscopic field | | | | Appearance | Movement |
|---|---|---|---|---|---|---|---|
| | | Control | Boiled extract | Solvents extract | Crude extract | | |
| 1 (24hrs) | 1 | 200 | 20 | 0 | 0 | Weak | Moving slowly |
| | $10^{-1}$ | | 50 | 0 | 0 | Weak | Moving slowly |
| | $10^{-2}$ | | 100 | 10 | 5 | Normal | Moving faster |
| | $10^{-3}$ | | 200 | 20 | 20 | Normal | Moving faster |
| 2 | 1 | 200 | 10 | 0 | 0 | Weak | Moving slowly |
| | $10^{-1}$ | | 20 | 0 | 0 | Weak | Moving slowly |
| | $10^{-2}$ | | 40 | 5 | 2 | Weak | Moving slowly |
| | $10^{-3}$ | | 100 | 10 | 8 | Normal | Moving faster |
| 3 | 1 | 200 | 5 | 0 | 0 | Weak | Moving slowly |
| | $10^{-1}$ | | 10 | 0 | 0 | Weak | Moving slowly |
| | $10^{-2}$ | | 20 | 0 | 0 | Weak | Moving slowly |
| | $10^{-3}$ | | 40 | 4 | 2 | Weak | Moving slowly |
| 4 | 1 | 200 | 0 | 0 | 0 | Weak | Moving slowly |
| | $10^{-1}$ | | 5 | 0 | 0 | Weak | Moving slowly |
| | $10^{-2}$ | | 10 | 0 | 0 | Weak | Moving slowly |
| | $10^{-3}$ | | 20 | 0 | 0 | Weak | Moving slowly |

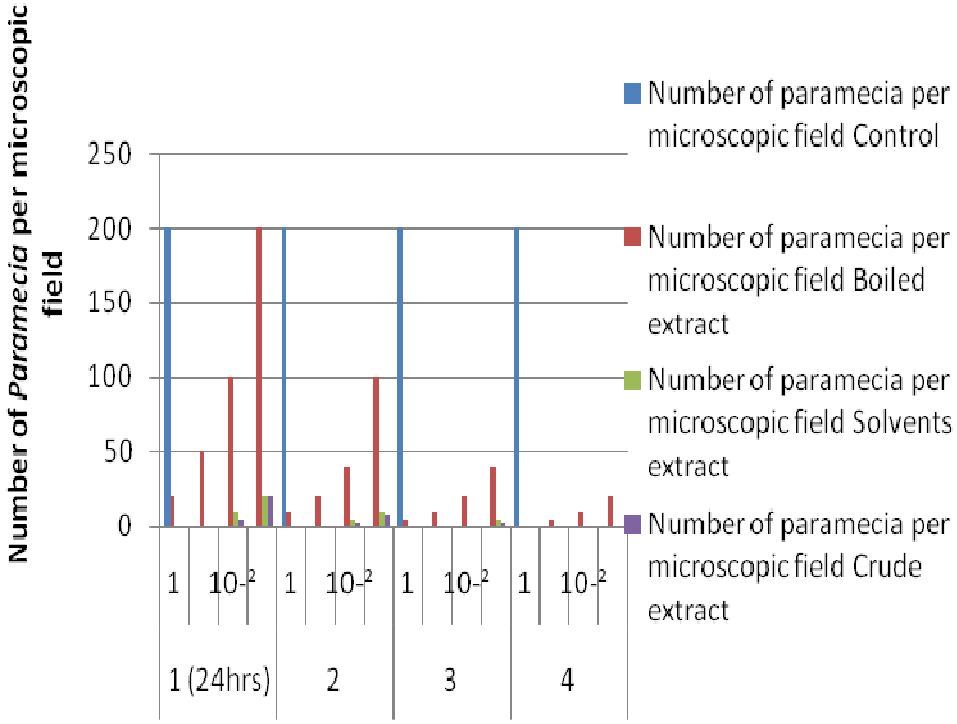


**Fig. 2. Anti-protozoal effects of *C. lusitanica* different extracts on *Paramecia***

### 3.3 Statistical Analysis

The SPSS (Statistical Package for Social Sciences) was used to analyze the results. The independent t-test values were calculated to compare the efficiency between *T. majus* and *C. lusitanica* and to compare the means of the number of the three different extracts versus to

the control. The relationship between concentration of extracts and the number of *Paramecia* was evaluated by using Chi-square.

#### 3.3.1 Relationship between different extract concentrations and the number of *Paramecia*

To see the association between *Paramecia* number with concentrations, grouping the number of *Paramecia* were made in two groups, (0-20 and 21-500), and then Chi-square was applied. The conclusion is shown by using suitable hypothesis.

$H_0$: There is no association between the number of *Paramecia* group and concentration of extracts.
$H_1$: There is association between the number of *Paramecia* group and concentration of extracts.
$H_0$: Null hypothesis.
$H_1$: Alternative hypothesis.

If $X^2$ calculated < $X^2$ tabulated, accept the null hypothesis.
If $X^2$ calculated > $X^2$ tabulated, accept the alternative hypothesis.

Since $X^2$ cal (6.603) < $X^2$ tab (7.8150), we accept $H_0$, and we conclude that there is no association between the number of *Paramecia* group and concentration of extracts.

#### 3.3.2 Comparison of the efficacy of *T. majus* and *C. lusitanica* extracts

The appropriate test to compare two mean/average measurements is Independent t-test.

Hypothesis:

$H_0$: $\mu_1 = \mu_2$
$H_1$: $\mu_1 \neq \mu_2$

If t calculated is < than t-tabulated, accept $H_0$
If t calculated is > than t-tabulated, accept $H_1$

This Independent samples test shows that the t-calculated is 1.170 as the t-tabulated at 5%, df=30 is 2.042, therefore accept the null hypothesis which means there is no true mean significant difference between the number of *Paramecia* after the application of extracts from the two plants.

### 3.4 Comparison of the Efficiency of the Extracts and the Controls

#### 3.4.1 Boiled extract versus control

Hypothesis:

$H_0$: $\mu_1 = \mu_2$
$H_1$: $\mu_1 \neq \mu_2$

If t-calculated is < than t-tabulated, accept $H_0$
If t-calculated is > than t-tabulated, accept $H_1$

This Independent samples test shows that the t-calculated is 3.27 as the t-tabulated at 5%, df=30 is 2.042, therefore accept the alternative hypothesis which means there is true mean significant difference between the number of *Paramecia* after the application of boiled extracts and the control.

#### 3.4.2 Solvent extract versus control

Hypothesis:

$H_0$: $\mu_1 = \mu_3$
$H_1$: $\mu_1 \neq \mu_3$

If t-calculated is < than t-tabulated, accept $H_0$
If t-calculated is > than t-tabulated, accept $H_1$

This Independent samples test shows that the t-calculated is 34.068 as the t-tabulated at 5%, df=30 is 2.042, therefore accept the alternative hypothesis which means there is true mean significant difference between the number of *Paramecia* after the application of solvent extracts and the control.

#### 3.4.3 Crude extract versus control

Hypothesis:

$H_0$: $\mu_1 = \mu_4$
$H_1$: $\mu_1 \neq \mu_4$

If t-calculated is < than t-tabulated, accept $H_0$
If t-calculated is > than t-tabulated, accept $H_1$

This Independent samples test shows that the t-calculated is 34.068 as the t-tabulated at 5%, df=30 is 2.042, accept the alternative hypothesis which means there is true mean significant difference between the number of *Paramecia* after the application of crude extracts and the control.

## 4. DISCUSSION

The extracts from two different plants known as *T. majus* and *C. lusitanica* showed varying anti-protozoal activity against *Paramecia*. The results visibly show the fact that soluble extracts of *T. majus* and *C. lusitanica* have anti-protozoal compounds and are able to inhibit the growth of *Paramecia*.

The result of methanolic extract against *Paramecia*, showed that the anti-protozoal activity of that extracts from *T. majus* recorded a considerable decrease in number of *Paramecia*. This means that hexane, methanol and chloroform solvents are suitable solvents for extracting anti-protozoal compounds in *T. majus* because all of the different concentrations used showed effects on the growth of the *Paramecia*. As the concentration of extract increased, the number of *Paramecia* decreased rapidly. However, the use of boiled extract showed slow changes in all concentrations. This means that boiling *T*. *majus*, is not a suitable mode of preparing remedy against protozoal infections mainly Trichomoniasis.

The effects of crude extract from *T. majus* against *Paramecia* showed that the number of *Paramecia* decreases quickly after application of the extract. This demonstrated that *T. majus* extraction by grinding it only without mixing with any solvent contributed to the powerful effect on *Paramecia* rather than the boiled one.

The anti-prototozoal effect in *T. majus* could be attributed to the Mustard-oils, Flavonols; kaempferol and quercetin present in the leaves, stems and roots. A glycoside found in the plant reacts with water to produce an antibiotic [11]. The German Commission E Monographs, a therapeutic guide to herbal medicine, approve *Tropaeolum majus* Nasturtium for urinary tract infections, cough, and bronchitis [12]. It is one of the herbs evaluated for safety and efficacy and sold in Germany. The plant is taken internally in the treatment of genito-urinary diseases, respiratory infections, scurvy and poor skin and hair conditions. Externally it makes an effective antiseptic wash and is used in the treatment of baldness, minor injuries and skin eruptions [13,14].

The anti-protozoal activity of the essential oils obtained from *C. lusitanica* was assayed against *Paramecia*. The results obtained from anti-protozoal screening method, followed by measurement of the minimum inhibitory concentration (MIC), indicated that *Paramecia* were the most sensitive microorganisms for this plant extracts. This sensitivity can be attributed to the components of the 5 fractions contained in extracts from *C. lusitanica* [6,14,15]. They have reasonably powerful anti-protozoal activity and that is why *Cupressus lusitanica* showed a powerful activity on *Paramecia*.

Extracts from *T. majus* and *C. lusitanica* was found to slow down the growth of *Paramecia* at different concentrations where increasing the concentration of extracts corresponded to the decrease of the number of *Paramecia*. This means that the quantity of the extracts is relative to the anti-protozoal activity because $X^2$ cal is 6.603 less than $X^2$ tab at 5% level df =30 is 7.815 and that there is no association between the number of *Paramecia* after application of the extracts and concentration of boiled extracts, but depending on the type of extract because the hypothesis was true for solvent and crude extracts from both plants.

When comparing the effectiveness of these two plant extracts on *Paramecia,* there is no significant difference between their reactivity*.* Statistical analysis helped to accept the null hypothesis because t-calculated is 1.170 less than t-tabulated at 5%, df=30 which is 2.042, this means that there is no true mean significant difference between the number of *Paramecia* after the application of extracts from the two plants. The susceptibility exhibited by *Paramecia* on these two medicinal plants, *T. majus* and *C. lusitanica* extracts, justified the use of these plants in the treatment of protozoa borne diseases like Trichomoniasis.

## 5. CONCLUSION

*T. majus* has been used in herbal medicine for respiratory and urinary tract infections [16]. The ethno medicinal use of *C. lusitanica* leaf oil in the treatment of whooping cough and skin infections has also been recorded [14]. In this study the extracts from both these two medicinal plants have been proven to contain anti-protozoal compounds that are capable of inhibiting the growth of *Paramecia*. This is proofed statistically as all t-calculated from all extracts compared to the controls showed a significant mean difference of the number of *Paramecia* between the control and the test panels after the application of the extracts.

Three solvents were used for *T. majus (*hexane, methanol and chloroform), where their extracting capacity helped to get methanolic extract, and one solvent (ethanol) was used for obtaining ethanolic extract from *C. lusitanica.* For both plants boiled and crude extracts were prepared to increase knowledge on the plants by comparing their different properties. Anti-protozoal activities of all extracts were tested against *Paramecia*. After several assays the counting of the number of *Paramecia* showed that the reactivity of both plants is almost the same. Crude and solvent extracts from both plants showed higher effects compared to the boiled extract which firstly increases the number of *Paramecia* before inhibiting them.

As the results of this study showed there is a strong possibility for the compounds in *T. majus* and *C. lusitanica* extracts to be utilized as an alternative antiprotozoal agent in the treatment of infectious disease such as Trichomoniasis. Increased discoveries related to *T. majus* and *C. lusitanica* will reduce high cost of modern drug for the treatment of Trichomoniasis infection and will reduce large economic loss caused by that damage. We hope future researches will continue in this line especially in the study of the potency of the isolated and purified active ingredient of these plants against to protozoa.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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