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The Effect of *Combretum molle* Seed Extracts on the Growth of the Mycelial Form of *Histoplasma capsulatum Var Farciminosum*-an *In Vitro* Trial

Kindu Wondmnew* **and Daniel Teshome***

DVM Wollo University, Dessie, Amhara, Ethiopia

***Corresponding authors:** Kindu Wondmnew, DVM Wollo University, Dessie, Amhara, Ethiopia, Tel: +251910749377; E-mail:* onbehaveofus@gmail.com

Daniel Teshome, DVM Wollo University, Dessie, Amhara, Ethiopia; Tel: +251913594781; E-mail: dteshome11@gmail.com

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Abstract

Epizootic lymphangitis (EL) compromises the welfare of working horses and has a serious negative impact on the livelihoods of cart horse owners/drivers in the affected parts of Ethiopia. Unfortunately, antifungal drugs for the treatment of EL are unavailable in both private and government clinics. The spread of multidrug-resistant strains of fungi motivates to discover new classes of antifungal compounds that inhibit these resistant strains. Therapeutic alternative compounds, particularly those isolated from plants have shown promising empirical effect on different fungal strains, which are unresponsive to chemical anti fungi drugs. *Histoplasma capsulatum var farciminosum* obtained from Aklilu Lema Institue of Patho Biology (ALIPB) was used as test organism. Agar dilution assay was performed to evaluate the inhibitory effect of *Combretum molle* seed extract and to measure the minimum inhibitory concentration (MIC). Ketaconazole was used as a positive control. *Combretum molle* seed extracts inhibit the growth of HCF up to 0.0156%. Hydrolysable tannins have a great potential in inhibition of fungal growth. In case of topical application, tannins have haemostatic effect and wound closure property.

Keywords: *Combretum molle* seed extract; Epizootic lymphangitis; Minimum Inhibitory concentration; Sabouraud dextrose agar

Introduction

Epizootic lymphangitis (EL) is a contagious, chronic disease, which mainly affects horses, mules, and donkeys. Epizootic lymphangitis (EP) is caused by *Histoplasma capsulatum var farciminosum* (HCF) which is a thermally dimorphic fungus. HCF was formerly described as an independent species, but this assessment has been changed and it is now considered a variety of *H. capsulatum* due to the close morphological similarities of both the mycelial and yeast forms [1]. The yeast forms of the fungus is found in the infected host and the mycelial saprophytic form in the environment [2,3].

The disease is characterized by a suppurative, ulcerating, and multifocal dermatitis, pyogranulomatous, and lymphangitis. It is seen most commonly in the extremities, chest wall and the neck, but it can also be manifested as an ulcerating conjunctivitis of the palpebral conjunctiva, or rarely as a multifocal pneumonia. The organism may also invade open lesions including ruptured strangle's abscesses and castration's wound [4].

The cutaneous form of the disease may be confused with farcy (the skin form of ganders), which is caused by *Burkholderia mallei*, ulcerative lymphangitis, which is caused by *Corynebacterium pseudotuberculosis*, indolent ulcers caused by *Rhodococcus equi*, *histoplasmosis* caused by *Histoplasm capsulatum var. capsulatum*, and strangles which is caused by *Streptococcus equi* [5,6].

The source of the *Histoplasma capsulatum var farciminosum* can be the skin lesions, nasal and ocular exudates of infected animals or the soil. Both the yeast form found in animals and the mycelial form found in the environment has produced Epizootic lymphangitis after experimental inoculation. In its saprophytic mycelial phase, *H. capsulatum* var. farciminosum, can survive for many months in warm and moist environments. This organism can also spread on fomites (common utensil) such as grooming or harnessing equipment. Biting flies in the genera *Musca* and *Stomoxys* are thought to spread the conjunctival form. Flies may also transmit the skin form mechanically when they feed on lesions and exudates. The pulmonary form probably develops when the animal inhales the organism [7].

The prevalence of the disease increases with assembling of animals. It was much more common when large numbers of horses were stabled together for cavalry and other transportation needs. Even though the disease mainly affects horses, mules and donkeys, the infection may occur in camels, cattle and dogs [1]. Experimentally, other animals have shown refractory to infection with the exception of certain laboratory animal species such as mice, guinea pigs, and rabbits [8,9]. Infection in humans has also been reported [10-12].

Many treatment types have been tried, but it was without success. Parenteral amphotericin B and iodides have been reported as effective. Even though Epizootic lymphangitis is highly prevalent and economically important in Ethiopia, the treatment options have not been employed because of the cost of the drugs and their absence in the market [13]. The current estimated cost of treatment with iodides for a single cart-horse is approximately 35USD which is not affordable to most cart horse owners. Besides to that, antifungal drugs for Epizootic lymphangitis treatment are not usually available in government and private veterinary clinics [14].

In non-endemic areas euthanasia of infected animals is recommended. However, in an endemic country like Ethiopia where socio-economic conditions are poor, a slaughter and eradication policy would not be acceptable [15]. Due to these Epizootic lymphangitis has a serious negative impact on the livelihoods of cart horse owners/

drivers in the affected areas and also compromises the welfare of working horses [14-16].

In Ethiopia, traditional medicine has long been practiced to treat some common ailments using plants available around them. Natural products and their derivatives have been historically sources of therapeutic agents [17]. Numerous plants are sources of antimicrobial agents, such as *combretum molle*, which used to treat numerous diseases in traditional way [18]. Antimicrobial compounds from plants may inhibit fungi through different mechanisms than conventional antibiotics and could therefore be of clinical value in the treatment of resistant microbes [19].

Combretum molle was used as a medicinal plant since ancient times [20]. The test of *C. molle* seed extract (CMSE) as antifungal property has been demonstrated in various studies [21]. Phytochemical studies carried out in the genus Combretum have shown the occurrence of many classes of constituents, including triterpenes, flavonoids, lignans, non-protein amino acid and tannins from different parts of the plant [22].

Combretum molle has been widely used as a medicinal plant to treat various diseases such as parasitic, protozoan, and fungal infectious diseases in East and West Africa [20]. Antifungal activity was reported in numerous fungal models that used *Candida albicans, Candida neoformans, Epidermophyton floccosum, Microsporum gypseum, Trichophyton mentagrophytes, Aspergillus fumigatus, Sporothrix schenckii and Microsporum canis* [21].

The above mentioned literatures are good indicators as CMSE will have a potential inhibitory effect on different fungi strains. However, no researches have done so far in the inhibitory effect of *C. molle* seed extracts for a convenient novel antifungal drug and for treatment of EL.

- Therefore, the objective of this study was to investigate the effect of CMSE on the growth of HCF and to determine minimum inhibitory concentration (MIC) of these extracts.
- To give highlight for future researcher about the effect of CMSE on HCF and for enabling they further study about this issue.
- To attract drug producers to formulate modern drugs from CMSE against HCF.

Materials and Methods

Study area

The study was conducted at the College of Veterinary Medicine and Agriculture of Addis Ababa University at Debre Zeit Campus between November 2013 and May 2014. Debre Zeit is located 47 km south east of Addis Ababa. The area has an altitude of 1860 meter above sea level with an average annual rain fall of 866 mm. It has bimodal rainy season; a main rainy season extending from June to September and short rainy season from March to May. The annual average minimum and maximum temperature is 11°C and 26°C respectively. Day length is constant throughout the year. Humidity of the area is about 50.9%.

Preparation of HCF for the test

All red grown *Histoplasma capsulatum var farciminosum* (HCF) was obtained from Aklilu lema Institute of Patho Biology (ALIPB). Then the sample was cultured into Sabourauds dextrose agar supplemented with 2.5% glycerol and 0.05% of chloramphenicol. Then after, the inoculated medium was incubated at 27°C for 21 days and the growth of the fungal colony was checked continuously once a week. Then the primary colonies were sub-cultured into Sabouraud's dextrose agar (SDA) slants (2.5% glycerol and 0.05% chloramphenicol) and incubated at 27°C for 3 weeks to get pure colony.

Plant used for the study

The seeds of *Combretum molle* were collected from the forests of Gonder, which is located in Amhara Regional Government, Northern part of Ethiopia. *Combretum molle* (velvet bush willow, local known as 'Abalo') is a tree with a larger, straighted trunk compared to most species of Combretum, further distinguished by its rough bark and dense crown.

Agar dilution assay

Preparation of extracts

The dried seed of *combretem molle* was crushed and ground in a grinding machine to obtain fine powder of the seeds. Ethanol extracts of *C.molle* seed were prepared according to the method of le Grand [23]. Briefly 50 gm of each powdered seed sample was mixed with 250 ml of 96% ethanol. The mixture was kept for 7 days in tightly sealed containers at room temperature and shacked several times daily. This mixture was filtered through filter paper to remove the coarse plant materials [24]. Further extraction of the residue was repeated 3 times until a clear supernatant extraction liquid was obtained. The filtrates of each tested plant were evaporated to dryness using a rotary evaporator at 40°C. The final dried samples were weighed and stored at room temperature until use.

Preparation of testing solution

Agar dilution assay (testing solution) is one of the methods used to test the antifungal effect of natural products [25]. Five grams of *Combretum molle* seed extract was mixed with 25 ml mixture of Di Methyl Sulfa Oxide (DMSO) and distil water to prepare a stock solution of 20%. From the first up to the last tube two ml mixture of DMSO and distil water was added. Two ml of 20% solution from the stock solution was transferred to the first test tube to prepare 10% solution. Two ml of 10% solution from the first test tube was transferred to the second test tube to prepared 5% solution. The procedures were continued by transferring two ml solution from 5% preparation to third test tube to get 2.5% concentration and continue in similar manner until 0.0024% was reached. Based on these procedures the following concentrations were prepared by using a mixture of DMSO and distil water (10%(4×10^{-1} gm), 5%(2×10^{-1} gm), 2.5%(1×10^{-1} gm), 1.25%(5×10^{-2} gm), 0.625%(2.5×10^{-2} gm), 0.313%($1.25\ \beta\ 10^{-2}$ gm), 0.156%(6.25×10^{-3} gm), 0.078%(3.13×10^{-3} gm), 0.039%(1.56×10^{-3} gm), 0.0195%(7.8×10^{-4} gm), 0.00975%(3.9×10^{-4} gm), 0.00488%(1.95×10^{-4} gm), 0.0024%(9.76×10^{-5} gm)).

Preparation of SDA media

The Saboraud dextrose media enriched by 2.5% of glycerol was prepared by dissolving SDA in distil water (in the ratio of 65 gram/litre), shaking well and heated for several minutes using stirrer hot plate to ensure complete dissolving of the media contents, then sterilized for 15 minutes at 1210c in an autoclave. After sterilization, the media was cooled to 500c in the water bath.

Two ml of Allred prepared serially diluted solutions of each extracts were mixed with 18 ml of SDA media (2.5% glycerol) to prepare media containing the following concentrations: 2%, 1%, 0.5%, 0.25%, 0.125%, 0.0625%, 0.0312%, 0.0156%, 0.0078%, 0.0039%, 0.0019%, 0.00098%, 0.00048%, and 0.00024%. After mixing well using a Vortex mixer, each media containing the dilution of extracts in universal bottles was inclined at 450 for 60 minutes to prepare slant in the bottle.

Preparation of control solution

For positive control, ketoconazole was used since amphotericin B was not available in the market. Ketoconazole was prepared in the range of 2×10^{-4}%, 1×10^{-4}%, 0.5×10^{-4}%, 0.25×10^{-4}%, 0.125×10^{-4}%, 0.062×10^{-4}%, 0.031×10^{-4}%, 0.015×10^{-4}%, 0.0078×10^{-4}%, 0.0039×10^{-4}%, 0.0019×10^{4}%, 0.00097×10^{4}%, 0.00048×10^{-4}%, and 0.00024×10^{-4}%. For the negative control, 10 ml of distil water was used as a stock solution.

Inoculums preparation

A pure sub-culture of the mycelia colony of HCF on SDA (with 2.5% glycerol and 0.05% chloramphenicol) was used for the tests. The colonies were scrapped by a sterile inoculating wire loop and transferred to a sterile saline (0.85%) solution. The turbidity of the suspension was adjusted by adding fungal colony or saline solution and vigorously mixed by using a Vortex mixer until turbidity approximates that of a 0.5 McFarland standard.

Inoculation of the media

The prepared SDA media mixed with the extracts and the control groups in different universal tubes were inoculated by dipping sterile swab into the test suspensions. After dipping into the suspension, the swab was uniformly streaked in the slant of the tube containing the SDA media in radial pattern. Finally, the lids of the inoculated agar plates were closed and incubated at 27°C 3-4 weeks with follow up [26].

Minimum inhibitory concentration

Minimum inhibitory concentration was defined as the lowest concentration of drug that completely inhibits the visible growth [27]. The MIC was determined after incubating the inoculated media at 27°C for 3 weeks. In order to determine the MIC, a fold of serial dilutions of SDA media containing ethanol extracts of *C. molle* seed, in the range of 0.00024% up to 2%, and ketoconazole (positive control), in the range of 0.0024×10^{-5}% up to 20×10^{-5} %, were prepared to inoculate with the test fungus. Distil water was used as negative control. The effect of the extract was determined by observing the mycelial growth in the inoculated media which contains different concentrations (2%, 1%, 0.5%, 0.25%, 0.125%, 0.0625%, 0.0312%, 0.0156%, 0.0078%, 0.0039%, 0.00195%, 0.00097%, 0.00048%, and 0.00024 %) of the seed extracts. The lowest concentrations that inhibit visible growth of the fungus after 3 weeks of incubation were considered as the MIC. The results of MIC were given in percentage (gram/ml) [28].

Data analysis

For this data we have not use any statistical software, instead we use SDA agar delusion test and we determine the minimum inhibitory concentration by taking the minimum concentration in which the fungus cannot grow after a continuous tests by different concentration.

Results

Effect of CMSE on the HCF

The results of ethanol extracts of *C. molle* seed extracts, by "the agar dilusion assay" are presented in (Table 1). The results shows that ethanolic extract of *C. molle* seed at concentrations of 2% 1%, 0.5%, 0.25%, 0.125%, 0.0625%, 0.0313% and 0.0156% have the inhibitory effect on *Histoplasma capsulatum var farciminosum*. However, 0.0078% concentration of the extract has no significant antifungal effect on *Histoplasma capsulatum var farciminosum* and it could not be able to prevent the growth of fungi on culture. The highest concentration of CMSE without growth inhibitory effects was 0.0078 %. The MIC of CMSE that inhibit the visible growth of HCF was 0.0156% (Figure 1).

Figure 1: Result of the agar dilution assay for CMSE which indicates the minimum inhibitory concentration i.e. 0.0156%, the dose of CMSE greater than 0.0156% does not permit the growth of HCF.

Histoplasma Capsulatum var Farciminosum was again sub cultured in Saboraud dextrose media containing the ketokonazole in the range of concentrations (2×10^{-4}%, 1×10^{-4}%, 0.5×10^{-4}%, 0.25×10^{-4}%, 0.125×10^{-4}%, 0.062×10^{-4}%, 0.031×10^{-4}%, 0.0156×10^{-4}%, 0.0078×10^{-4}%, 0.0039×10^{-4}%, 0.0019×10^{-4}%, 0.00097×10^{4}%, 0.00048×10^{-4}%, 0.00024×10^{-4}%). The MIC of the ketokonazole (positive control) against HCF was 0.25×10^{-4} %. Fungal growth was not observed in media containing the positive control in the concentration of (2×10^{-4}, 1×1^{-4}%, 0.5×10^{-4}% and 0.25×10^{-4}%). The highest concentration of positive control (ketkonazole) without growth inhibitory effects was 0.125×10^{-4}% (Figure 2). The results of the agar dilution assays are summarized in table 1.

Macroscopical appearance of *Histoplasma capsulatum var farciminosum*

The sub culture of mycilia of HCF on SDA (2. glycerol) grew initially as glabrous or wrinkled and creamy (whitish to whitish brown in colour), dry and convoluted colonies measuring 4-14 mm in diameter after 21 days of incubation at 27°C. The result gives the appearance shown in figure 1.

Microscopical appearance of *Histoplasma capsulatum var farciminosum*

Lacto phenol cotton blue wet mounts prepared from portions of the sub-cultured colonies revealed branching hyphae and chlamydo spore

upon examination under microscope (Figure 3). A Giemsa stained smear made from these colonies and examined under microscope revealed mycelia cells with double cell surroundings (Figure 4).

ketoconazole		***C.molle* seed extracts**	
Concentration %	**Growth**	**Concentration %**	**Growth**
2%	-	2×10^{-4}%	_
1%	-	1×10^{-4}%	-
0. 5%	-	0.5×10^{-4}%	-
0.25%	-	0.25×10^{-4}%*	-
0.125%	-	0.125×10^{-4}%	+
0.0625%	-	0.0625×10^{-4}%	+
0.0312%	-	0.0312×10^{-4}%	+
0.0156%*	-	0.0156×10^{-4}%	+
0.0078%	+	0.0078×10^{-4}%	+
0.00390%	+	0.00390×10^{-4}%	+
0.00195%	+	0.00195×10^{-4}%	+
0.00097%	+	0.00097×10^{-4}%	+
0.00048%	+	0.00048×10^{-4}%	+
0.00024%	+	0.00024×10^{-4}%	+
0%	+	0%	+
*=MIC-=no visible growth +=visible growth			

Table 1: Growth of HCF in different concentrations of C.molle seed extract and ketoconazole.

Figure 2: Result of the agar dilution assay of ketoconazole which indicates the minimum inhibitory concentration of the well-known antifungal drug ketoconazole that used for comparison for CMSE efficacy.

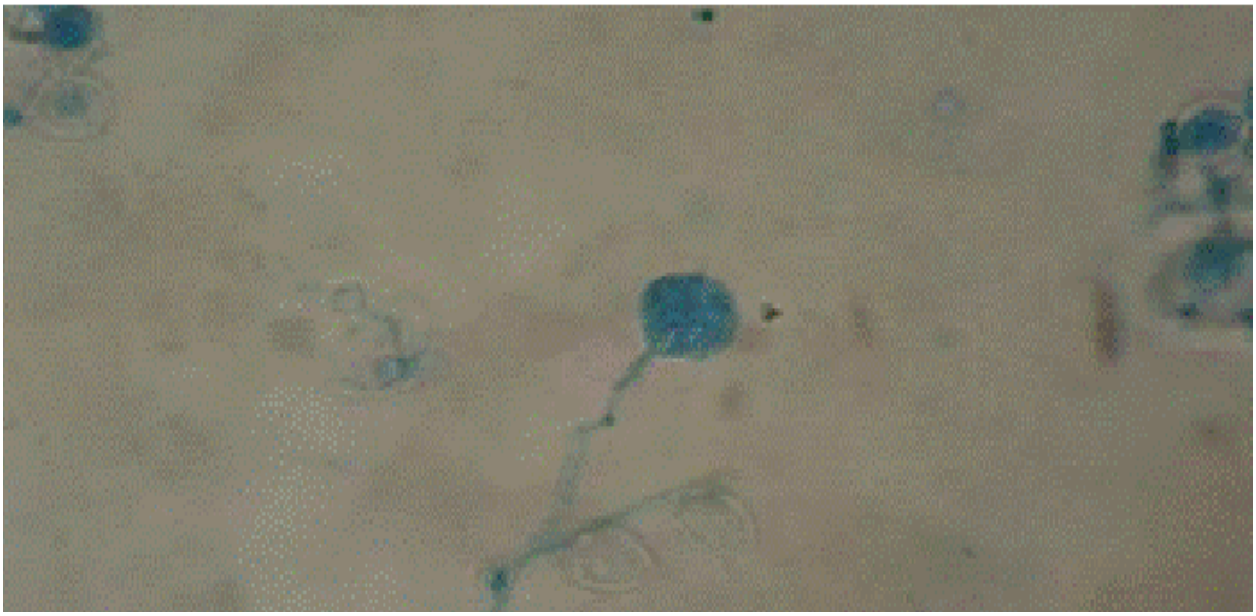

Figure 3: Branching hyphae and chlamydospore in a lactophenol cotton blue preparation made from mycelial colonies of HCF cultured on SDA (2.5% glycerol and 0.005% chloramphenicol).

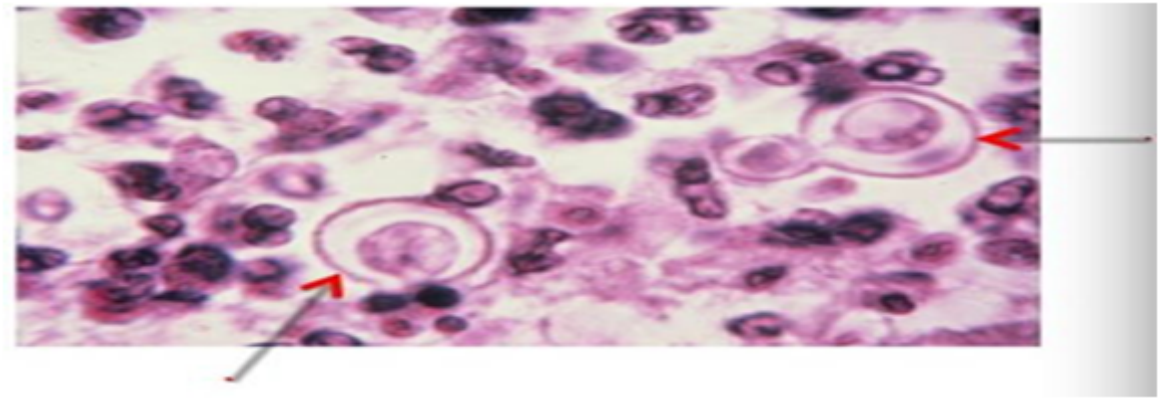

Figure 4: Giemsa-stained smear made from colonies of HCF. The red arrow points a double walled HCF cell.

Discussions

The morphological picture and macroscopical characteristics of the grown HCF colonies on SDA media were resembled to the original samples obtained from the laboratories of ALIPB. Both new colonies on SDA media and preserved colonies of HCF had macroscopic appearance of glabrous or wrinkled, creamy (whitish to whitish brown in colour), dry and convoluted. During microscopic appreciation of the HCF colony under gram- stained smear made by staining the colony, it was appeared as purple (Gram-positive) and lemon shape. In the culturing process of HCF, laboratory accustomed HCF grew well in a shorter period of time (21 days) than HCF taken directly from lesion of EP which take 6-8 weeks at 27°C.

The MIC of CMSE obtained in this study was 0.0156 %. However, the difference between the MIC (0.0156%) and the maximum none inhibitory concentration (0.0078%) suggests that the MIC could be lower. As [29] stated the MIC for novel pharmacological compounds should be <0.1%. So the MIC (0.0156%) is below 0.1% and hence this extract could be considered active. The positive control Ketaconazole was found to be more potent than CMSE in inhibiting the growth of the mycelia form of HCF [30].

The result obtained in this study was an indication that CMSE has inhibitory effect on HCF. The Minimum inhibitory effect of CMSE obtained from present study is also in harmony with previous studies made on fresh garlic extract (0.5 mg/ml, 0.05%) by [31], *phytolaca dodecandra* (0.03%) by [32]. In a work conducted by [32], the MICs of n-butanol and aqueous extracts of *Phytolaca dodecandra* were (0.039%-0.078%) and (0.625%-1.250%), respectively. The difference in MIC of the two extracts of *Phytolaca dodecandra* could be ascribed by the difference in the polarity of the solvent used in the extraction

process. This was supported by [21,33,34], as the polarity of the solvent has great effect on the quantity and types of bio-molecules extracted.

In the search of efficient and cost effective antibiotics for the control of infectious disease due to the ineffectiveness of conventional antibiotics [35-37] the solvent has great effect. The main antifungal molecule in *Combretum molle* seed extract is tannin [18]. The presence of phenolic hydroxyl groups on the surface of tannin molecules participate strongly in the biological activities of tannins. It combines with protein and other polymers to form stable complexes through nonspecific forces such as hydrogen bonding, hydrophobic effects and covalent binding [38]. This is done by hydrolysis of ester linkage between gallic acid which eventually affects the biosynthesis of cell wall and cell membrane. Impairment of biosynthesis of cell wall and cell membrane cause to increase the permeability of cell membrane and alterations of Cell wall .This leads to decrease cell volume and disjunction of cell membrane from the cell wall [39]. This leads to leakage of internal contents and no more exchange of molecule between cell wall and cell membrane.

As [40] tried to explain, tannins have two forms, and these are hydrolysable and condensed tannins which affect fungal growth. In the same study made by [37], both Hydrolysable and condensed tannins have been found to possess antifungal effect. But the hydrolysable tannins were found to be more effective against fungi. This is because Hydrolysable tannins (gallic acid and ellagic acid) are linked to esters of core molecules which will be hydrolyzed easily while condensed tannins are not susceptible to hydrolysis [40]. In other research done by indicated that the fungicidal effect of the extract was due to the presence of high amount of hydrolysable tannins.

In addition to its fungicidal effect, when CMSE is use topically it will promote tissue healing, stop bleeding, stop further infection and heal the wound internally. As mentioned by [41] the ability of tannins to form a protective layer over the exposed tissue keeps the wound from being infected even more.

Conclusion and Recommendations

The investigation of chemical compounds from natural products is fundamentally important for the development of new drugs. In the extraction process of active chemicals from plant the solvent used for maceration determines both the quality and quantity of molecules that will be produced. Ethanol macerated CMSE have shown a promising anti-fungal effect on mycelial form of HCF. The MIC of CMSE that inhibited the growth of HCF is ≥ 0.0156%. The main anti-fungal molecule in *C.molle* seed extract is hydrolysable tannins. The main action of tannin on HCF is inhibition of cell wall and cell membrane biosynthesis. In topical application tannin has haemostatic and wound closure effect. The result indicates that *C.molle* seed can be used for the treatment of EL if convenient methods of preparation, dose, and route of administration are established through meticulous in vitro and in vivo trials. Based on the trials that have done, the following studies should be done in the next trends;

- Further isolation of the active chemicals to synthesis new drugs.
- Identify the broad-spectrum activity of the extracts.
- Study the mechanism action of the extracts and their toxic effect on lab animals.
- Use more polar compounds to extract active chemicals.

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