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PURIFICATION OF KERATINASE FROM *TRICHOPHYTON RUBRUM* AND STUDY THE INHIBITORY EFFECT OF SOME AQUEOUS PLANTS EXTRACTS ON THE PURIFIED ENZYME GROWTH

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Abstract

The study aims to purify the keratinase enzyme that produced from the local isolation of *Trichophyton rubrum* by steps, including concentrated it by ammonium sulphate with 30% saturation ratio, dialysis, and finally by gel filtration technique using Sephadex G-75, The number of purification times was 9.1 with an enzyme yield of 30%, The fungus was grown for (5, 10, 15 days), The enzyme activity was then measured. The highest activity was recorded at 10 days which amounted of 61.5 (U/ml). The effect of some plants (*Aloe Vera, Rhuscoriaria, Hot Pepper, Beta Vulgaris, Prunusarmeniaca*) on the fungal growth and keratinolytic activity was studied in *Trichophyton. rubrum* as one of the major etiologic agents of human and animal dermatophytosis in Salah Aldin - Iraq and other parts of the World. To investigate an alternative antidermatophyte by some plant extracts as a natural product on keratinase activity which was a virulence factor of *T. rubrum*. Culture conditions for 25 isolates of *Trichophyton. rubrum* which was isolated from human Dermatophytosis in both sexes with ages of 1-60 years at Dermatology Advisory Unit of Salah Aldin General Hospital. The aqueous extractions of (*Aloe Vera, Rhuscoriaria*, *Hot Pepper*, *Beta Vulgaris*, *Prunus armeniaca*) at various concentrations (10, 20, 30, 40 mg/ml) inhibited the growth of *Trichophyton rubrum*, This inhibition reached to a maximum level at 40% concentration of extracts. The Keratinase activity for *Trichophyton rubrum* isolate has been 61.5 (U/ml) Of the five extracts used in the study, the extract of *Aloe Vera* plant was the best in reducing the effectiveness of keratinase enzyme 43.5(U/ml) in concentration 10 (mg/ml) and Keratinase activity was also inhibited by the extract about 33 (U/ml) in concentration 40 (mg/ml).

Keywords: (*Aloe Vera*, *Rhuscoriaria*, *Hot Pepper*, *Beta Vulgaris*, *Prunus armeniaca*), Keratinase, *Trichophyton. rubrum*.

Introduction

Dermatophytes are Keratinophilic fungi capable of invading keratinized tissues of human and animals because Keratinophilic skin, hair and nails by ability to synthesize an extracellular enzymes called keratinases, causing dermatophytosis (Lee *et al.,* 2014). Keratinase is an extracellular enzyme used for the bio degradation of keratin. It is produce only in the presence of keratin substrate and The chemical classifications of keratinase (Ec. 3.4.99.11) Al-Musallam *et al.,* (2013) and its type of Keratinases are proteases belong to the group of serine hydrolases that are capable of degrading keratin, a fibrous and insoluble structural protein extensively cross-linked with disulfide, hydrogen and hydrophobic bonds Keratinases are proteolytic enzymes which play very important role in the invasion of keratinized tissues and have mostly studied in the dermatophyte *Trichophyton rubrum*, the world wide fungi in distribution and have both anthropophilic and zoophilic forms (Chinnapun, 2015). Over the years, a number of hypotheses have been proposed to explain the mechanism of keratin degradation by microbial keratinases. Broadly speaking, it is agreed that keratin degradation encompasses two main stages: deamination and keratinolysis while keratin degradation in keratinolytic fungi also includes an additional mechanical step involving the frond myceliain dermatophytes and boring hyphae in non-dermatophytes that penetrate the substrate surface, The keratinolytic activities of dermatophytes are higher than the non-dermatophytes. Amongst non-dermatophytic fungi, soft

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keratin degraders are likely to produce thin boring hyphae and hard keratin degraders tend to form swollen boring hyphae. In addition to sulphur-containing amino acids, sulphite is also produced by dermatophytes from environmental cysteine, a process that is governed by the key enzyme cysteine dioxygenase Cdo1, which is then secreted by the sulphite efflux pump Ssu1. As keratin is rich in cysteine, the mechanism of cysteine conversion and sulfite efflux may also play a role in keratin degradation (Grumbt *et al.,* 2013). The Cdo is a virulence factor, crucial for keratin degradation, as it is involved in the oxidation of cysteine to cysteine sulphinic acid during disulphide bridges cleavage (Kasperova *et al.,* 2013). The Discovery of antimicrobial activities of (*Aloe Vera, Rhuscoriaria*, *Hot Pepper*, *Beta Vulgaris*, *Prunus armeniaca*) have a long history and it is reported on different microorganisms such as fungi, bacteria and virus and these plants are the most used plants in the medical field where it works as an ointment, the inhibitory effects of aqueous extracts due to Several chemical compounds shown the antifungal properties of these plant against dermatophytes and other fungi because of its components, As for *Beta vulgaris* it contains alkaloids compounds which Cinnamic acid 'Choline' Chenoabicine 'Betaine' Isorhamnetin n-ferulouaspartate) and flavonoid compounds (Quercetin, Hispidulin) And proteins and lipidis Naglaa (2015). While *Prunus armeniaca* it contains Alkaloids and glycosides (Tuhfa *et al.,* 2017).

Material and Methods

Collection of samples

Dermatophytes: 25 isolates of *Trichophyton rubrum* were used in this research which isolated from patients with dermatophytosis by making scraping during routine diagnostic works in Salah Ad-Din General Hospital during March to July 2017 from both sexes their ages range from 1-60 years. These isolates were identified based on colony and microscopic morphology, urease test, hair perforation test and the test of growing in temperature at 25°C for 10 days.

Extraction and purification of keratinase

Purification steps of keratinase included: extraction of the enzyme, the precipitation of the enzyme by ammonium sulphate, dialysis, Gel filtration chromatography by using phosphate buffer saline PBS 0.2 M and pH 9, and gel filtration by using Sephadex G-75 gel.

Extraction of keratinase

The keratinase enzyme was extracted from the selected isolates *Trichophyton rubrum* after grown in the optimal conditions for the enzyme production and perform the process of harvesting the cells using centrifuge at 6000 r.p.m for 20 minutes and get a clear solution, It's followed by subsequent purification steps.

Ammonium sulphate Precipitation

Ammonium sulphate was used in the deposition process of the raw enzymatic extract at different saturation ratios (10- 60%). These different ratios were selected separately until the best saturation ratio was obtained.

Dialysis

Dialysis tube was activated by immersing in boiling distilled water (D.W.) for few minutes, the precipitate solution (50 ml) resulted from ammonium sulphate fractionation (10-60%) was poured into the dialysis tube, and then dialysis tube was incubated in a baker containing 500 ml of phosphate buffer saline 0.1 M in the refrigerator at 4°C for 48 hours. The sample was concentrated by embedding the dialysis tube within sucrose powder for 30 minute.

Gel filtration chromatography

Preparation and packing of the gel

The gel prepared according to the instructions of the company equipped with Pharmacia Fine Chemicals, 10 g of Sephadex powder was suspended in 250 ml of distilled water and heated in a 90°C water bath for 3 hours Then left to be cooled and washed with phosphate solution twice, then suspended with an appropriate amount of the same solution and the deairing process was conducted. The separation column with dimensions of (1.2 × 30 cm) was used, which contains the Sephadex G-75 gel, was used to separate the protein compounds. The gel was placed at a height of 20 cm by gently pouring it on the column walls to prevent air bubbles from forming. The column was then washed with distilled water and the column was ready to pass the model and The solution of the deposition after the dialysis process of processing for *Trichophyton rubrum* fungi is Phosphate-buffered saline solution with a concentration of (0.2) molar and a pH 9.

Add models

The precipitate solution was used in the separation column containing the gel (Sephadex G-75), with 2 ml of concentrated solution followed by washing with 2 ml of Phosphate-buffered saline solution. The enzyme was recovered at a flow rate of 30 ml / h by 3 ml for one part the enzymatic content was studied by reading the absorption intensity at the wavelength (280) nanometer and using the UV-spectrophotometer. The specific enzyme activity was then collected. The enzymatic components of the isolated fungi were collected separately and the

process was repeated several times to collect sufficient quantity of the parts.

Implantation and extraction

The production media of keratinase enzyme prepared by method of Gradisar *et al.,* (2005) of Ammonium chloride, with concentration of 0.5 g/L magnesium hydrochloride 0.1 g./L sodium chloride 0.5 g/L, potassium monohydrogen phosphate 0.3 g/L and bi-hydrogen potassium phosphate 0.4 g/L, yeast extract 0.1 g/L the above ingredients were dissolved in 800 ml of distilled water and adjust pH to 9. The base material then added, it is a horns powder and the volume completed to a liter of distilled water and then distributed in volumetric flasks of 250 ml and 50 ml of mentioned media, the media was sterile by autoclave at a temperature of 121°C for 15 minutes, after leaving the flasks to cool the production media of liquid enzyme has inoculated by adding a tablet with 10 mm diameter by Corkscrew with 7 mm diameter from the edge of the fungal colony, with 7-10 days age of isolated fungus, cultivated in the agriculture media prepared for fungus development, enzyme production and pH control for the phosphorus solution used in the media on pH 9 prior to inoculation, The flasks then were incubated in quickly vibrate incubator at 120 rpm at 30°C for 10 days. After the incubation period, Mycelium and the remains of the horns and the non-decomposing materials were separated by centrifugal extraction at a temperature of 4°C and at a speed of 5000 cycles/min for 10 minutes. The leachate was separated from the precipitate through the filter paper of Wattman No.1 type. The solutions was used as a crude extract for the subsequent steps, The enzyme effectiveness and protein concentration were estimated in the leachate containing the crude enzymatic extract.

Estimation of the protein concentration and effectiveness of keratinase

According to the method described by Metodo Biuret, The concentration of keratinase in the leachate of Fungal Culture was estimated according to the enzymatic method mentioned in the kit analysis of the French company (Maizy). And The activity Enzyme activity was estimated on the basis of the degradation of keratin protein and the release of amino acids such as Tyrosine by enzyme action by adding 0.5 keratin powder in 100 ml of 0.028 molar potassium phosphate precipitate at pH 9 to 0.5 mL of enzyme solution and incubation at 45°C for 30 min and then stop the reaction by adding 1 ml of acetic acid trichlorine solution (Trichloro acetic acid (TCA) 10%). The protein method was estimated according to the method described by Metodo Biuret.

Preparation of aqueous Extracts

The aqueous extract was prepared by plant grinding with an electric mill; the 40 g of the grinded plant was then weighed. The 160 ml of sterilized distilled water was added. The mixture then Mixed into the electric mixer and leave the mixture in the fridge for 24 hours for the purpose of soaking. It was then filtered through several layers of filter papers (Wattman No.1) to remove the non-grinded plant parts and the remaining fibers, then pour the extract in sterile glass dishes, put in an oven at a temperature not exceeding 40°C until the entire water evaporates and the extract then put after dry in glass bottles with a tight lid and kept in the refrigerator until using then, 1 g of dry plant extract and solvent was taken in 10 ml of Sterile distilled water. The solution was become at concentration of (100 mg/ml). This extract was sterilized using Millipore filters with a diameter of 0.22 Mm. This standard concentration was considered as a source for the preparation of the subsequent solutions used in the study (Salah *et al.,* 2013)**.** General chemical detection of active compounds in plants extracts used in the study have been identified and analyzed through qualitative and quantitative detection of chemical compounds using high pressure liquid chromatography (HPLC) this analysis was conducted at the Ministry of Science and Technology / Baghdad. Stationary phase column with the phenomenex type and the minute size is 3 Mm was used. The mobile phase is acetic acid at a flow velocity of 1 ml/min at 30°C using ultraviolet radiation along a 245 nm wave length Where 0.1 g of sample dissolved in 5 ml aqueous solution of methanol, the centrifugation was performed at a speed of 7500 rpm for 15 minutes and the pure suspension was treated with charcoal to remove the dyes and was dried and then re-suspended in 0.1 ml of methanol by the mixer, The mixture was passed through a single-use filter and 20 µL of the model was injected into the HPLC (Amani, 2012).

Results

Dermatophyte

Trichophyton rubrum isolates were separated from skin scales of dermatophytic patients after culturing of the specimens on Sabourand Dextro's Agare Media. The isolates were identified based on the production of powdery or cottony white-cream colonies, microscopic features as Hypha divided into several large Conidial with sceptrum shape, divided by several barriers between range of (5-8) and small barriers in many numbers and in a tearful form.positive results in urease, hairperforation tests and the ability of growing at 25°C and 7 days.

General chemical detection of active compounds

in plant extracts

The results of the chemical detection of some active substances of The Terpene, Tannin and volatile oils were found in the *Rhus coriaria*, while in *Aloe Vera* plant contain the following compounds: Terpene and glycoside while Alkaloids and glycosides were found in the *Prunusa rmeniaca*, Terpene and Capsaicin in hot pepper, proteins and lipids in *Beta Vulgaris*, as shown in the Fig. (1, 2, 3, 4, 5).

Extraction and purification of keratinase

Crude enzyme

The results of the current study revealed that the crude enzyme solution had a total protein concentration of 3.1mg/ml and an enzyme activity of 0.3 (µ mole/ml)

Ammonium sulphate fractionation

The keratinase was precipitated by ammonium sulphate with 10-60% saturation percentage as a first step of purification, the results of this step revealed that the protein concentration was 3.9mg/ml while the enzyme activity was 0.5µmole/ml at the best Satisfaction ratio 30 %.

Dialysis

The results of this step revealed that the protein concentration was 2.2mg/ml while the enzyme activity was 0.6µmole/ml.

Gel filtration chromatography by using Sephadex G-75 gel column

The enzyme was further purified using gel filtration and the product of dialysis (51 ml solution) was applied to Sephadex G-75 gel. column (Fig. 6). This demonstrated

Table 1: Purification steps of keratinase of *T. rubrum*.

Extract	10(mg/ml) extract conc.	40(mg/ml) extract conc.
Aloe Vera	33(U/ml)	43.5(U/ml)
Rhus coriaria	34.5(U/ml)	57(U/ml)
Hot Pepper	54(U/ml)	57(U/ml)
Beta Vulgaris	37.5(U/ml)	54(U/ml)
runus armeniaca	40.5(U/ml)	52.2(U/ml)

Table 2: The effects of aqueous plants Extractions on keratinase activity.

Volume (ml)	Activity (µ mole /ml)	Protein (mg/ml)	Specific activity (U/mg)	Total activity (U)	Yield (%)	Purification fold
100	1	30	0.12	3.1	0.3	100
50	1	15	0.12	3.9	0.5	30
40	2.25	12	0.27	2.2	0.6	20
30	9.1	9	1.1	0.9	1	9

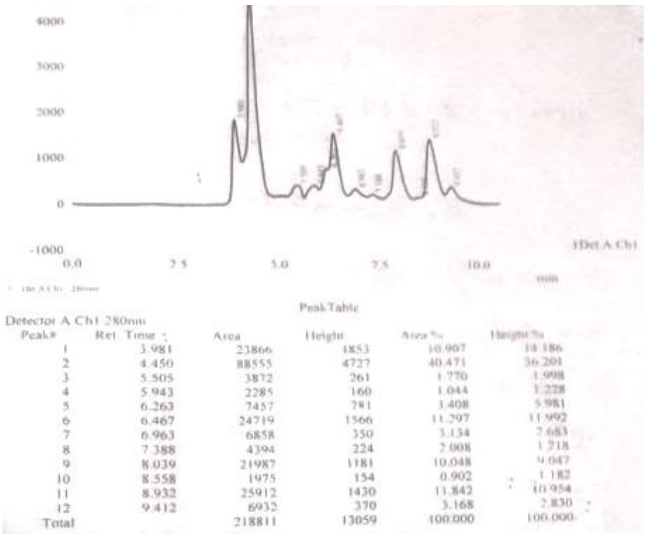


Fig. 1: Hplc of Tannins in *Rhus coriaria*

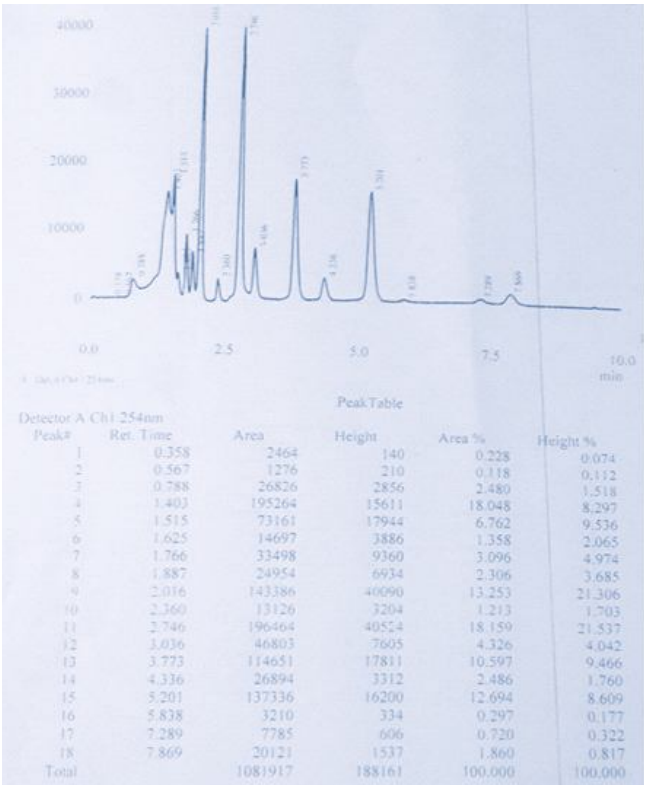
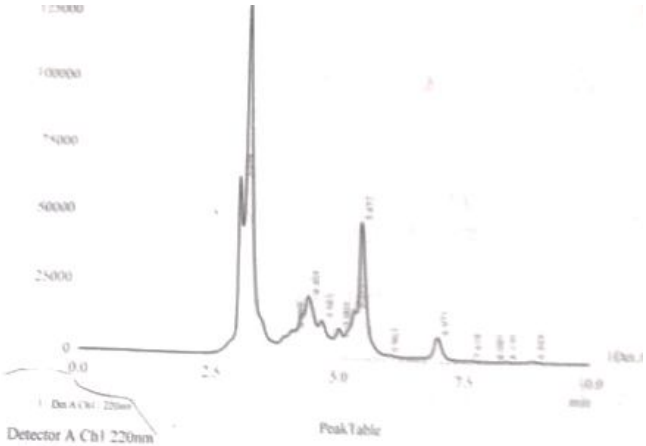


Fig. 2: Hplc of Glycoside compound in *Aloe Vera*

that single peak of protein was observed with a concentration of 0.9 mg/ml and the fractions number 31 through 41 correlated with that peak were collected and showed an enzyme specific activity of 1 µ mole/ml (Table 1).

Effects of aqueous plants extract on fungal growth and keratinase activity

The results showed that fungal growth was inhibited by (*Aloe Vera*, *Rhus coriaria*, *Hot Pepper*, *Beta Vulgaris*, *Prunus armeniaca*) extracts. These inhibitions

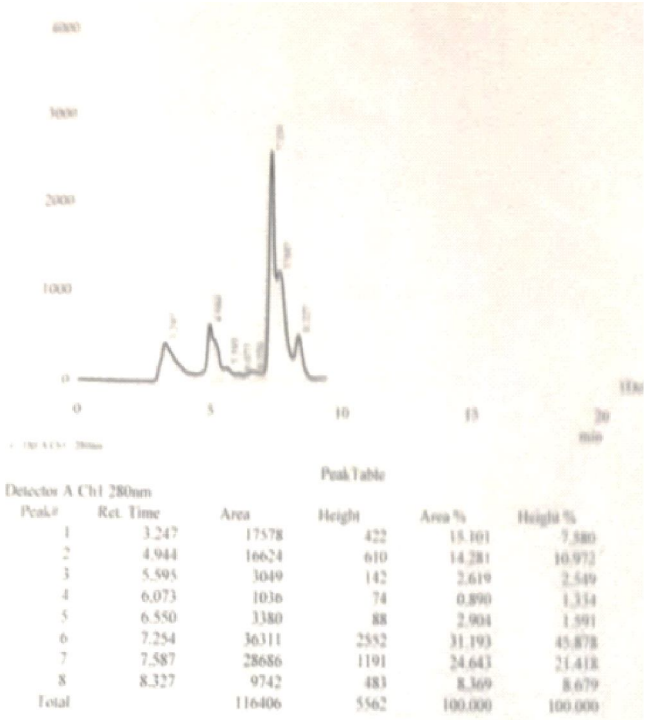


Detector A Ch1 220nm

PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.054	435267	62930	12.407	19.527
2	3.233	1302474	129991	37.127	40.337
3	4.099	157008	9011	4.475	2.796
4	4.401	389640	21001	11.107	6.517
5	4.661	166834	12776	4.756	3.965
6	5.001	111198	10166	3.170	3.155
7	5.317	190402	16648	5.427	5.166
8	5.472	546140	48396	15.568	15.018
9	5.967	1267	62	0.036	0.019
10	6.971	146233	8541	4.168	2.650
11	7.628	22491	846	0.641	0.262
12	8.080	5490	531	0.156	0.165
13	8.330	11614	577	0.331	0.179
14	8.888	22122	788	0.631	0.245
Total		3508181	322265	100.000	100.000

Fig. 3: Hplc of *Prunus armeniaca*



Detector A Ch1 280nm

PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.247	17578	422	15.101	7.580
2	4.944	16624	610	14.281	10.972
3	5.595	3049	142	2.619	2.549
4	6.073	1036	74	0.890	1.334
5	6.550	3380	88	2.904	1.591
6	7.254	36311	2552	31.193	45.878
7	7.587	28686	1191	24.643	21.418
8	8.327	9742	483	8.369	8.679
Total		116406	5562	100.000	100.000

Fig. 4: Hplc of Capsaicin in hot pepper

were significant as compared with the controls which was shown in the Fig. (7, 8, 9,10, 11). The keratinase activity was also inhibited by these extracts. The *Aloe Vera* extract is the beast, which was shown in table 2 at the lowest concentration 10(mg/ml) caused 43.5(U/ml)

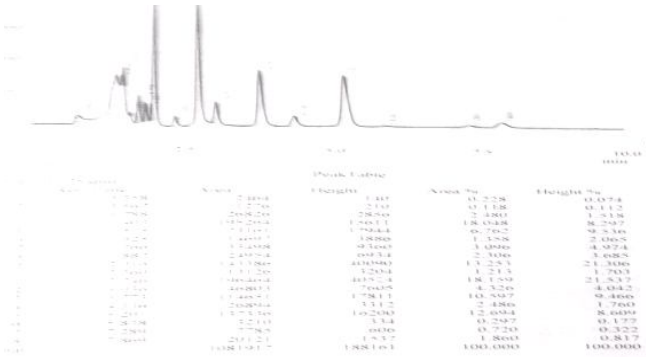


Fig. 5: Hplc of *Beta vulgaris*

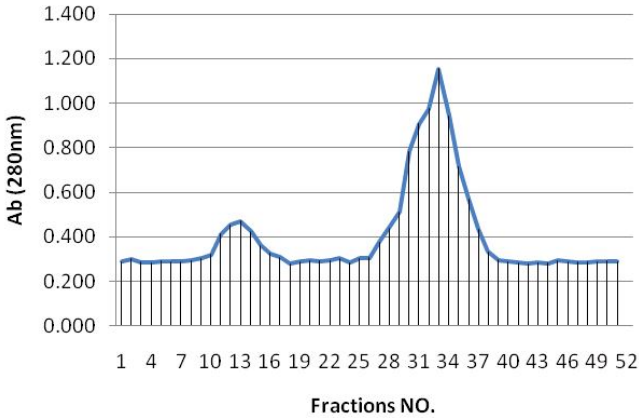


Fig. 6: The Purification of keratinase by gel filtration chromatography using Sephadex G-75column (1.2×30) cm. The column calibrated with phosphate buffer saline PBS 0.2 M and pH 9, flow rate 30 ml/hour.

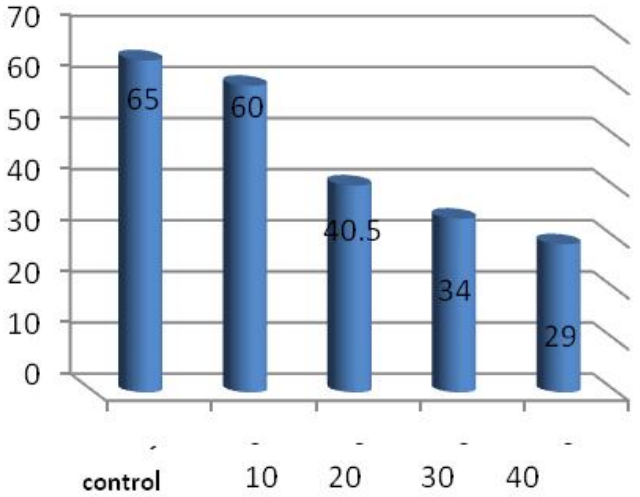


Fig. 7: Effects of *Aloe Vera* aqueous on *T. rubrum* growth

in enzyme activity by the fungus. The maximum inhibition of keratinolytic activity of 40 (mg/ml) concentration of *Aloe Vera* extract was 33 (U/ml) as shown in the fig 12.

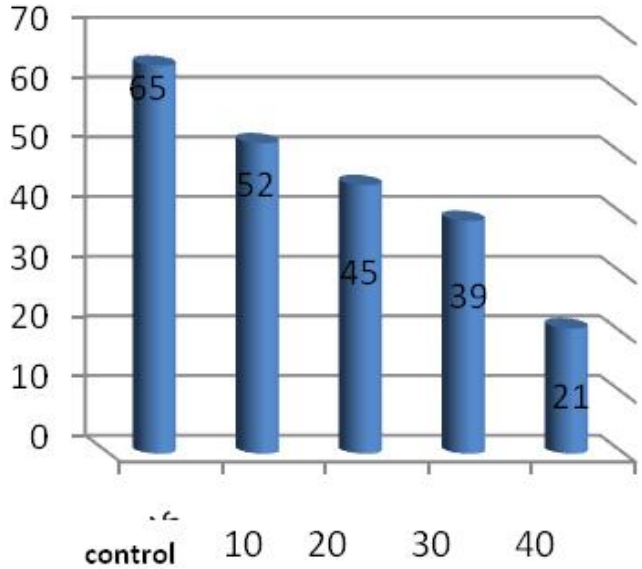


Fig. 8: Effects of *Rhus coriaria* aqueous on *T. rubrum* growth

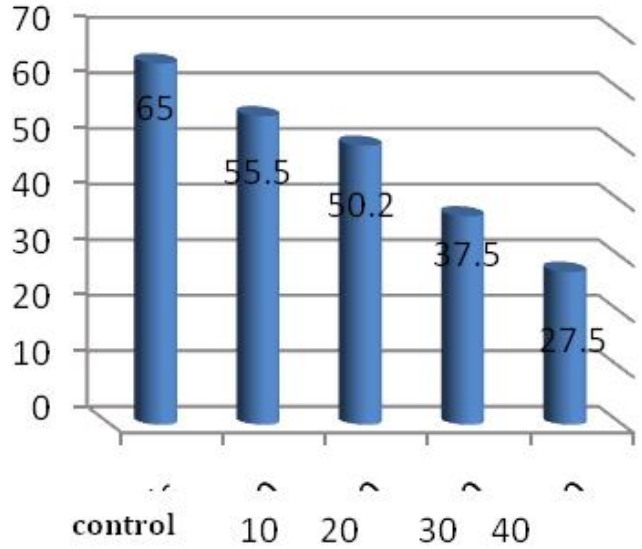


Fig. 9: Effects of *Beta vulgaris* aqueous on *T.rubrum* growth

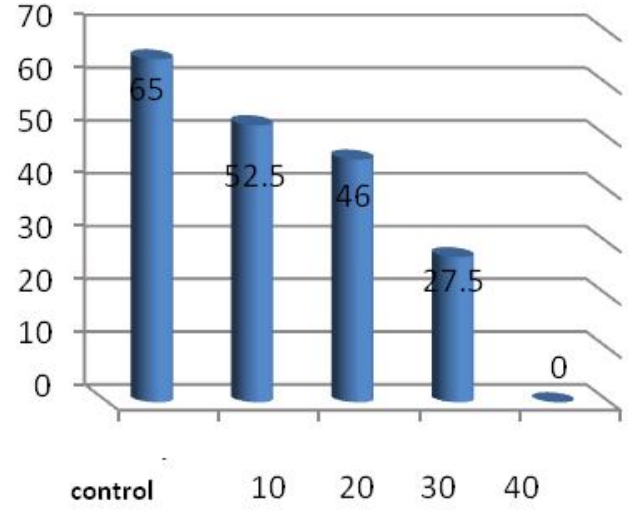


Fig. 10: Effects of *Hot Pepper* aqueous on *T. rubrum* growth

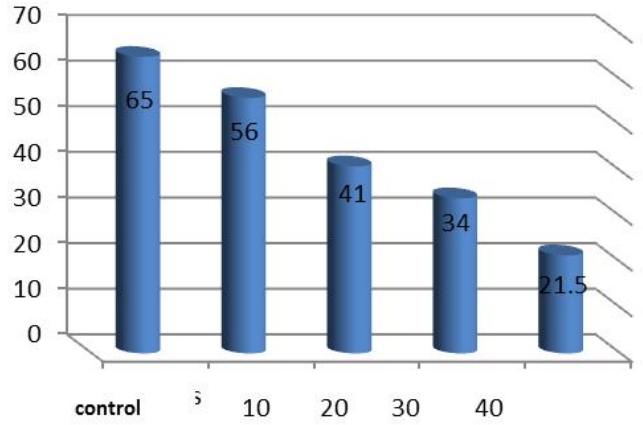


Fig. 11: Effects of *Prunus armeniaca* aqueous on *T. rubrum* growth

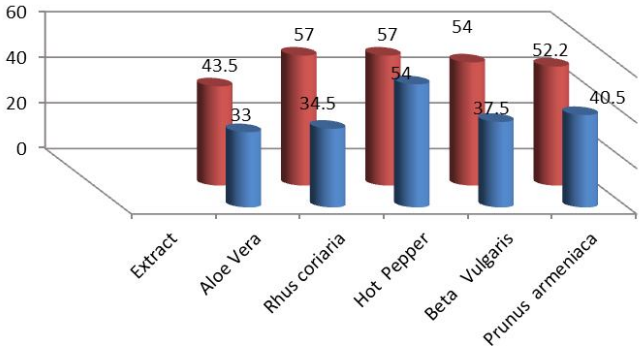


Fig. 12: Effects of aqueous plants Extractions on Keratinase activity of *Trichophyton rubrum*.

Discussion

The dermatophytes divided into three mainly genus which are *Microsporum*, *Epidermophyton* and *Trichophyton*. The fungus *Trichophyton rubrum* considered the most common world wide in the infection of dermatophytosis. One of the reasons that led to the spread of this species to the rest of the species because it generates a small immune response with large numbers of mycelia and the possession of the pathogen for some Lipases enzymes, including phospholipase (Kadhim *et al.,* 2015). Ammonium sulphate is one of the most salts used in the concentration of enzymes for their high yield and low cost, compared to other organic solvents, lack of effect on pH and enzyme stability, and no change in enzyme composition. The concentration of ammonium sulphate depends on the principle of the equivalence of the charges on the surface of the protein and the degradation of the surrounding water layer, leading to its deposition by the effect of the known Salting out. Anbu *et al.,* (2005) showed that the optimum saturation rate of ammonium sulphate was 70% for the concentration of keratinase from *T. rubrum* fungi; it has been obtained 3.2 times purification, with a 58.5% enzymatic yield. In another study, keratinase produced from *Scopulariopsis*

brevicaulis was concentrated in ammonium sulphate by 80% saturation Chromatography of gel filtration The chromatography experiment was performed using the Sephadex G75 column with dimensions (1.5 × 30 cm) in the purification process. The dispersion of the deposition solution from the dialysis process through the Sephadex G-75 column. The washing and recovery parts were collected individually and the enzymatic activity was estimated in each of them, the parts containing the highest enzymatic activity were mixed. The specific activity for *Trichophyton rubrum* fungi was 1.1 u/mg Protein, with purity of 9.1 times and with an enzyme yield of 30% In present study, The inhibitory effect of studied plant extracts is due to their containment of tannins, Terpenes, Resins, capsin and volatile oils, which are known as Antibiotics, disinfectant materials, sterile materials and toxic oxidants material for microorganisms (Khalil *et al.,* 2009). Glycosides inhibit the enzymes and transport proteins that present in the cell membrane and proved its ability to some microbes, including fungi by effect on living components such as mitochondria, and thus lead to the poisoning and cells death while Tannins act to precipitate the proteins associated with the cellular membranes of fungi and thus affect the process of entry and exit of substances into and out of the fungal cell as well as their formation a complexities with polysaccharides and their effect on enzymes (Kumar *et al.,* 2012). Terpenes are non-nitrogenous chemical compounds, which are a volatile oil derivative and it is characterized by being anti-fungal While the inhibitory activity of the turbines represents by its ability to tear the cellular membranes by Lipophilicity compounds, Capsin is also an effective as chemical anti-inflammatory agent for fungal pathogens and a natural antioxidant (Al-Kattan and Al-Ishlash, 2012). The most important one is Our work on Keratinase Activity that aqueous *Aloe Vera* extract is disrupts hypha cell wall and causes massive necrosis and disarrangement in some cellular compartments specially nucleus and mitochondria in *Trichophyton rubrum* Thus, This may be due to the mechanism of action of the active compound found in *Aloe Vera* plant. These compounds are glycosidic : Aloin is and Barba lion, Aloe-emodin Ibtisam, (2016) while The Terpenes are non-nitrogenous chemical compounds, a volatile oil derivative and have anti-fungal properties Jeyasakthy *et al.,* (2017). As it works to tear the cellular membranes by Lipophilicity compounds, these compounds affect the enzyme locations of the fungal cell build proteins and enzymes. and may affect the metabolic activity of fungi through their effect on the cells Osmotic pressure of the fungus leading to shrinkage and the accumulation of cells cytoplasm, and thus cell death. As for flavonoids, they disrupt or work break up cell membranes by forming complexes with extracellular dissolved proteins (Renisheya *et al.,* 2012). *Aloe Vera* contains antiseptic agents: salicylic acid, urea nitrogen, cinnamon acid, phenols and sulfur. all have the inhibitory action on fungi, bacteria and viruses (MuazAhmeda, 2013). The gel also contains an anti-inflammatory magnesium lactate, which helps prevent itching, and salicylic acid and other ant prostaglandin compounds, which relieve inflammation. The leaf lining (latex, resin or sap) contains glycosides (aloin, aloe-emodin and Barba loin) (Bhagyasri *et al.,* 2018).

Conclusions

Fungal growth and keratinolytic activity are important factors in pathogenesis of the dermatophytes, their inhibition by (*Aloe Vera*, *Rhus coriaria*, *Hot Pepper*, *Beta Vulgaris*, *Prunus armeniaca*) indicate that this substance may have potential values for treatment of human and animal dermatophytosis.

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Conflict of Interest

The authors declare that they have no conflict of interest in the publication

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