

## EVALUATION OF *MYRTUS COMMUNIS* FLAVONOID AS ANTIDERMATOPHTIC AND KERATINASE INHIBITOR

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### ABSTRACT

This study was applied for extraction flavonoid compounds from *Myrtus communis* (aerial part) and detection of its effectiveness on growth of one of dermatophytic isolates and keratinase activity, *Trichophyton mentagrophytes* was the most common isolates which was obtained with frequency (66%), the identification and quantitation of flavonoids was carried out by thin layer and high performance liquid chromatography. The results showed that each 100 g of aerial part of the plant contained 850 mg total flavonoids as rutin). The HPLC chromatogram for *M. communis* flavonoid extract revealed that, *M. communis* flavonoid extract contained Rutin, Quercetin, Apigenin, Kaempferol, Luteolin. The inhibitory effect of flavonoid against *Trichophyton mentagrophytes* and keratinase activity was evaluated. The higher effect was observed at concentration 10 mg/ml against fungal isolate. The minimum inhibitor concentration was at 3mg/ml while the higher inhibition of keratinase activity was obtained at concentration 5mg/ml with 25% remaining enzyme activity.

Keywords: HPLC, fungi, enzyme activity, inhibition zone.

عباس وآخرون

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تقييم الفلافونويدات من نبات الاس كمضاد لل Dermatophyte ومثبط للكيراتينيز

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المستخلص

اجريت هذه الدراسة بهدف استخلاص مركبات الفلافونويدات من نبات الاس (*Myrtus communis*) و التحري عن تأثيرها في نمو احدى عزلات الفطريات الجلدية كما درس تأثيرها على فعالية انزيم الكيراتينيز. كان الفطر (*Trichophyton mentagrophytes*) هو الاكثر ترددا و بمعدل 66% عن بقية العزلات. تم الكشف والتقدير الكمي عن الفلافونويدات بتقنية كروماتوغرافيا الطبقة الرقيقة وكروماتوغرافيا السائل عالية الدقة. اظهرت النتائج ان كل 100 غرام من النبات احتوت على 850 ملغرام فلافونويدات كلية معبر عنها كمركب الروتين. كما اظهرت نتائج كروماتوغرافيا السائل عالية الدقة لمستخلص الفلافونويدات من نبات الاس احتواء المستخلص على مركبات (الروتين، كوارستين، ابجنين، كامبفيرول ولوتيونين). قدر التأثير التثبيطي للفلافونويدات المستخلصة ضد عزلة *Trichophyton mentagrophytes* وفعالية انزيم الكيراتينيز، لوحظ اعلى تأثير كان عند تركيز 10 ملغرام امل ضد نمو العزلة الفطرية و التركيز المثبط الادنى كان 3 ملغرام امل بينما كانت اقل فعالية متبقية لانزيم الكيراتينيز 25% عند التركيز 5 ملغرام امل.

الكلمات المفتاحية: كروماتوغرافيا السائل عالية الدقة، فطريات، فعالية انزيم، قطر التثبيط

## INTRODUCTION

Dermatophytosis poses a serious problem to backward population. Superficial infections caused by keratinophilic fungi are called ringworm infections or tinea infection, Dermatophytes are pathogenic fungi that have the ability to degrade and paralyse the keratin membrane of the skin due to their ability to produce proteolytic enzymes, which it uses to hydrolyze keratin, the main protein constituent of hair, nails, feet and skin(34,36). *Microsporum*, *Epidermophyton* and *Trichophyton* are the three genera of this group. There are about 40 species in these three genera(16,28). *Trichophyton* is the commonest dermatophyte which is geophilic, zoophilic and anthropophilic in nature. The genus *Trichophyton* includes 24 species, some of these are saprophytes. The most common human pathogenic species of *Trichophyton* genus are *Trichophyton mentagrophytes*. In many parts of the world *T. mentagrophytes* is isolated most frequently. *T. mentagrophytes* is typically found in moist, carbon-rich environments(6). Although a large number of synthetic allopathic drugs are available in the market. The majority of these clinically used antifungals suffer from various drawbacks in terms of toxicity, lack of fungal efficacy cost and emergence resistant strains caused by the frequent use of them. In recent years, there has been an increasing search for new antifungal compounds due to the lack of efficacy, side-effects and or resistance associated with some of the existing drugs (6,7) Traditional medicine is useful and without any side effect like the chemical medicine. Various phytoconstituents have been reported to be present in plants which are antidermatophytic (31). Myrtus is medicinal plant that has been used worldwide in traditional medicine. Myrtus species contain phenolic acids as gallic and ellagic acids, flavonoids, myricetin, quercetin, catechin, so have been found in leaves and stems of *M. communis*, fatty acids, tannins and Anthocyanin pigments(2). Flavonoids consist of a large group of polyphenolic compounds having a benzo- $\gamma$ -pyrone structure and are ubiquitously present in plants. They are synthesized by phenyl propanoid pathway. flavonoids are thought to have health-promoting properties due to their high antioxidant capacity both *in vivo* and *in vitro* systems (22,8).

Flavonoid rich plant extracts from different species have been reported to possess anti activity against a wide array of microorganisms (11,25,26). Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesions, enzymes, cell envelope transport proteins, and so forth. Lipophilic flavonoids may also disrupt microbial membranes (4,23,29). The present work aimed to evaluate the antidermatophytic and keratinase inhibition by *M. communis* flavonoids.

## MATERIALS AND METHODS

### Fungal isolates

All the isolates of *Trichophyton mentagrophytes* were isolated from skin scrapings from 60 patients admitted to Dermatology Department of Baghdad Educational Hospital suffering from ringworm disease. The fungal cultures of *T. mentagrophytes* were isolated and maintained on Sabourouds Dextrose agar medium and identified using both macroscopic and microscopic conventional standard methods such as, hair perforation, urease activity, growth in PDA medium. Sabourouds Dextrose Agar (SDA) was used for the culturing and maintenance of fungal isolates(20,7,3).

### Collection of plant material

The (aerial parts) of *Myrtus communis* were collected from University of Baghdad/Iraq area, and were classified in the faculty of college of Agriculture Engineering Sciences-University of Baghdad. Plant parts were collected and washed in running tap water to remove dust. They were dried at room temperature (25 °C) and manually grinded, then packaged in sterile containers and kept away until use.

### Extraction of total flavonoids

The total flavonoids of *Myrtus communis* was obtained by macerating 60g of powdered plant sample (areal part) with D.W contain 10% (v/v) HCl, refluxed for 8 hours, the extract was filtered and re-extracted with ethyl acetate (1:1 v/v) the fraction was washed with distilled water to neutrality and dried. The total flavonoid was determined according to method (14,15,30), using Rutin as standard, the extract was analysed by means of Thin layer chromatography.

**Detection of flavonoid by Thin layer chromatography:** Thin layer chromatography was

carried out using standard methods for qualitative assay and quantitative assay using Rutin standard curve according to (23,18). Stock solution of sample (5mg/ml) was dissolved in respective solvents. Standard included Rutin, Quercetin, Kampherol and luteolin were dissolved in ethanol, the mobile phase which used for flavonoid separation was (chloroform :glacial acetic acid: formic acid ) (44:3.5:2.5). The plate (Silica gel Aliminum 60 sheet) after drying was visualized directly with UV at 254 nm, the Rf value of the different spots that observed was calculated.

#### **Detection of flavonoid by HPLC**

The detection of flavonoid in plant extract was achieved using HPLC under following conditions. HPLC was performed on a shimadzu technologies modular model (LC-2010 Ashimatzu/Japan). The analysis was carried out using ODS<sub>c</sub>18 column (250x4.6 Id) mm, 5mm particle size, the mobile phase was Methanol 65% : 35% (1% Nitric acid), the flow rate was 0.8 ml/min. The UV detector monitored at 340nm, while the injection volume was 20 µl (35).

#### **Production of keratinase from *Trichophyton mentagrophytes* using submerged culture**

The keratinase production medium prepared according to Allpress, Lin (21,1), the medium contained, per liter of D.W the following: (NH<sub>4</sub>Cl 0.5g, NaCl 0.5g, K<sub>2</sub>HPO<sub>4</sub> 0.3g, KH<sub>2</sub>PO<sub>4</sub> 0.4g, MgCl<sub>2</sub>.6H<sub>2</sub>O 0.1g, yeast extract 0.1g, chicken feather 10g), pH 7.5, fungal suspension 1% (3\*10<sup>6</sup> spore/ml) was cultured in 250 ml capacity Erlenmeyer flask containing 50 ml of sterilized medium. Flasks were incubated at 28 °C for 7 days. At the end of growth period the cultures were centrifuged at 6000 run/min for 15 min at 4 °C then the supernatant used for further analysis of keratinase assay.

#### **Keratinase assay**

Keratinase activity was measured according to Allpress (21). The reaction mixture containing 0.5 ml of substrate solution (keratin 5% dissolved in 0.028 M phosphate buffer pH 8) with 0.5 ml of crude enzyme solution incubated at 45 °C in a water bath for 30 min. The reaction was stopped by adding 1 ml of 10% (w/v) trichloroacetic acid and centrifuged (10000 rpm, 15 min). The absorbance of the supernatant was measured at 280 nm. The blank was

treated in the same way except for the addition of TCA before the initiation of enzyme reaction. The keratinase activity was expressed as one unit of the enzyme corresponding to an increase in the absorbance value (0.1).

#### **Antifungal activity**

On sterile plates containing Sabouraud dextrose agar, the fungal cultures were swabbed, then wells of 6 mm diameter were bored in each plate. The wells were filled with flavonoid extract which prepared with (5, 10 mg/ml) concentration by dissolved 0.05, 0.1 g in 10 ml D.W. separately the plates incubated at 28 °C for 7 days. After end of incubation the diameter of inhibition zones formed around the wells was measured in millimeter (7). Two control prepared the first (negative control well filled with D.W only) and the second (positive control well filled with klorancazole 75 mg dissolved in 1 ml D.W.) for comparison.

#### **Determination of minimal inhibitory concentration *in vitro***

Minimal inhibitory concentrations (MIC) of *Myrtus communis* flavonoids against *T. mentagrophytes* were determined, series concentrations of flavonoids were prepared at different concentrations (3, 5, 7, 9, 10 mg/ml), plates were prepared and cultured as in determination antidermatophytic activity with triplicate for each concentration. Two control prepared (negative control well filled with D.W only) and (positive control well filled with klorancazole 75 mg dissolved in 1 ml D.W.) for comparison. The minimum concentration of the extract which inhibited fungal growth was considered the minimum inhibitory concentration (MIC).

#### **Effect of *Myrtus communis* flavonoid on keratinase activities**

The ability of *Myrtus communis* flavonoids to inhibit keratinase activity was evaluated using the method of Costa (9). Cell-free supernatant (0.5 ml) was mixed with 0.5 ml of flavonoid extract (using different concentration 3, 5 and 7 mg/ml) and incubated for ten minute at 37 °C, after the incubation, 0.5 ml of each reaction mixture above was added to 0.5 ml of substrate solution (5%) separately, then incubated at 45 °C in water bath for 30 minute. Then reaction was stopped by adding 1 ml TCA 10%, the reaction solution centrifuged at 10000 rpm for 15 min. Enzyme activity was estimat-

ed and the blank was prepared by adding 1ml of TCA 10% to substrate solution then the crude enzyme was added with 0.5 ml flavonoid extract 3 mg/ml concentration. The control reaction did not contain flavonoid and was performed exactly the same as the experimental reactions for estimation remaining activity of enzyme as %percentage.

## RESULTS AND DISCUSSION

### Isolation of *Trichophyton mentagrophytes*

**Table1. Dermatophyte species isolated from patients with dermatophytosis**

| Dermatophyte species               | number    | Percentage% |
|------------------------------------|-----------|-------------|
| <i>Trichophyton mentagrophytes</i> | 40        | 66.6        |
| <i>Trichophyton rubrum</i>         | 12        | 20          |
| <i>Microsporum canis</i>           | 8         | 13          |
| <b>Total</b>                       | <b>60</b> | <b>100</b>  |

The fungal infection of skin and scalp (dermatophytosis) considered a common problem especially in the tropical and subtropical regions of the world where warm and humid climate provides a favorable environment for fungi (5). They have become a significant health problem affecting children, adolescents, and adults (16).

**Extraction and detection for total flavonoids:** Each 60g powdered aerial part of *M. communis* yielded 1.7g residue. The result of TLC analysis for total flavonoids of *M. communis* showed the presence of two spots under UV light at 254nm, With Rf value 0.61, 0.71. Depending on standards, the results indicated that this extract contained Quercetin and Luteolin (figure1). The quantitative determination of total flavonoid depending on standard curve of Rutin revealed that each 100g aerial part of the plant contained 850 mg total flavonoid as Rutin. HPLC applied for identification flavonoids of *M. communis* by comparison with the standards (depending on retention time and the absorbance spectrum), the HPLC chromatogram for *M. communis* flavonoid extract revealed the presence of nine peaks (Figure2.), by comparison with retention time for some standards flavonoid (Figure3.), results indicated that, *M. communis* flavonoid extract contained Rutin, Quercetin, Apigenin, Kaempferol, Luteolin with retention time 4.387, 5.594, 6.600, 8.236, 9.061 min respectively. HPLC is the successor of TLC. In TLC separations it becomes difficult to differentiate

From 60 samples which diagnosed as dermatophytosis the most common isolates were *T. mentagrophytes* with rate (66%) Table1 which gave positive results with microscopic examination and showed ability to penetrate the hair and showed ability to changing the color of urea test media from yellow to pink to give positive result and gave negative result with growing on PDA media.

between overlapping bands and spots. In contrast the peaks in HPLC can be easily resolved and evaluated by controlling operational parameters such as flow rate of mobile phase, buffer control of the mobile phase, column oven temperature, The differences of flavonoids content for *M. communis* crude extract depending on *Myrtus* species. Also final extract composition depends on extraction solvent used for extract preparation, mainly because of its polarity. Moreover the contents of total flavonoids vary among myrtle parts(2,17,19).



**Figure 1. Thin layer chromatography TLC Chromatogram (for *M. communis* total flavonoids extract) on Slica gel Aliminum 60 sheet with mobile phase chloroform: glacial acetic acid: formic acid with standard solutions including: Rutin(R), Kaempferol(K), Luteolin(L), Quercetin(Q).**

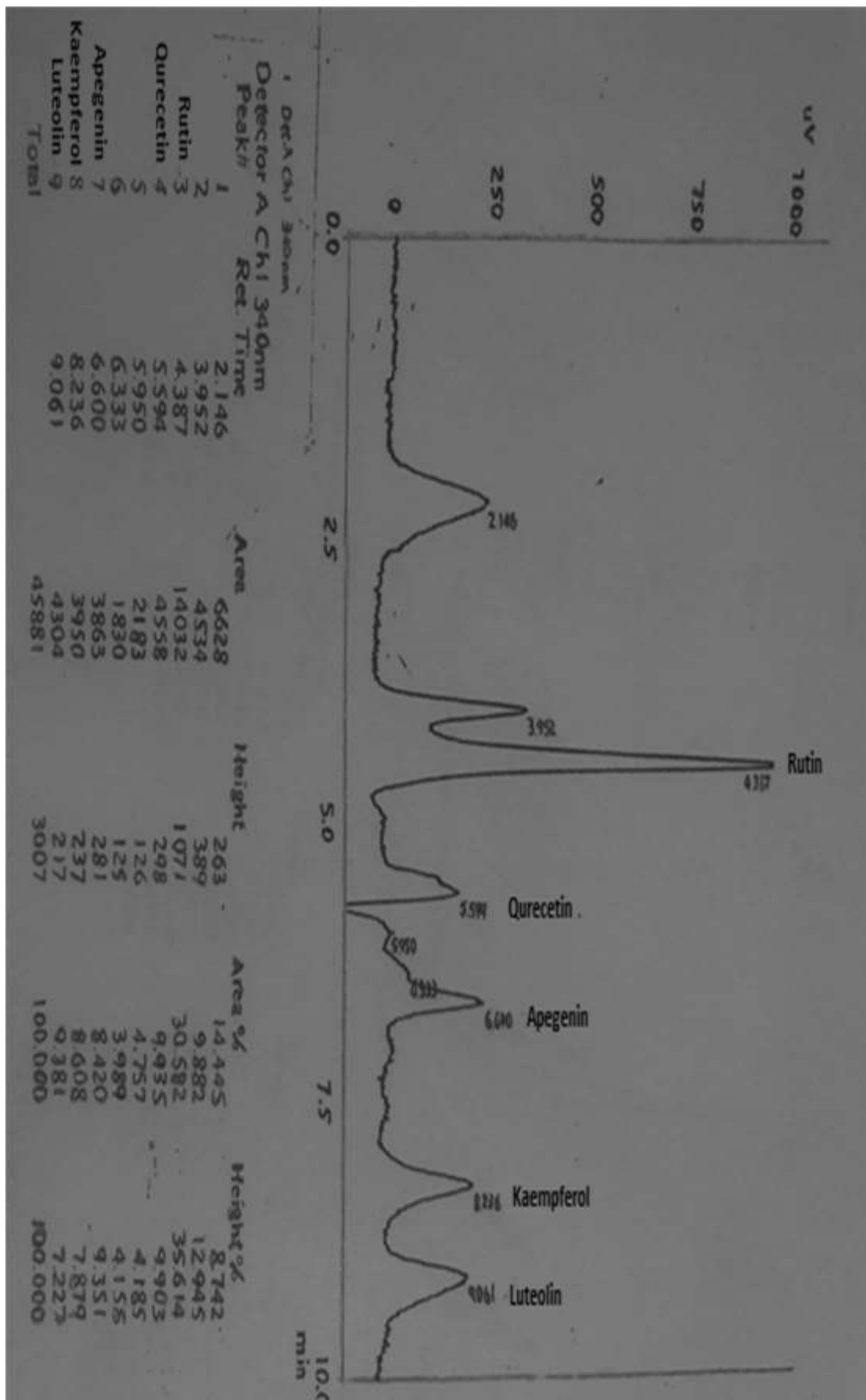


Figure2. HPLC Chromatogram of the flavonoids in *Myrtus communis*

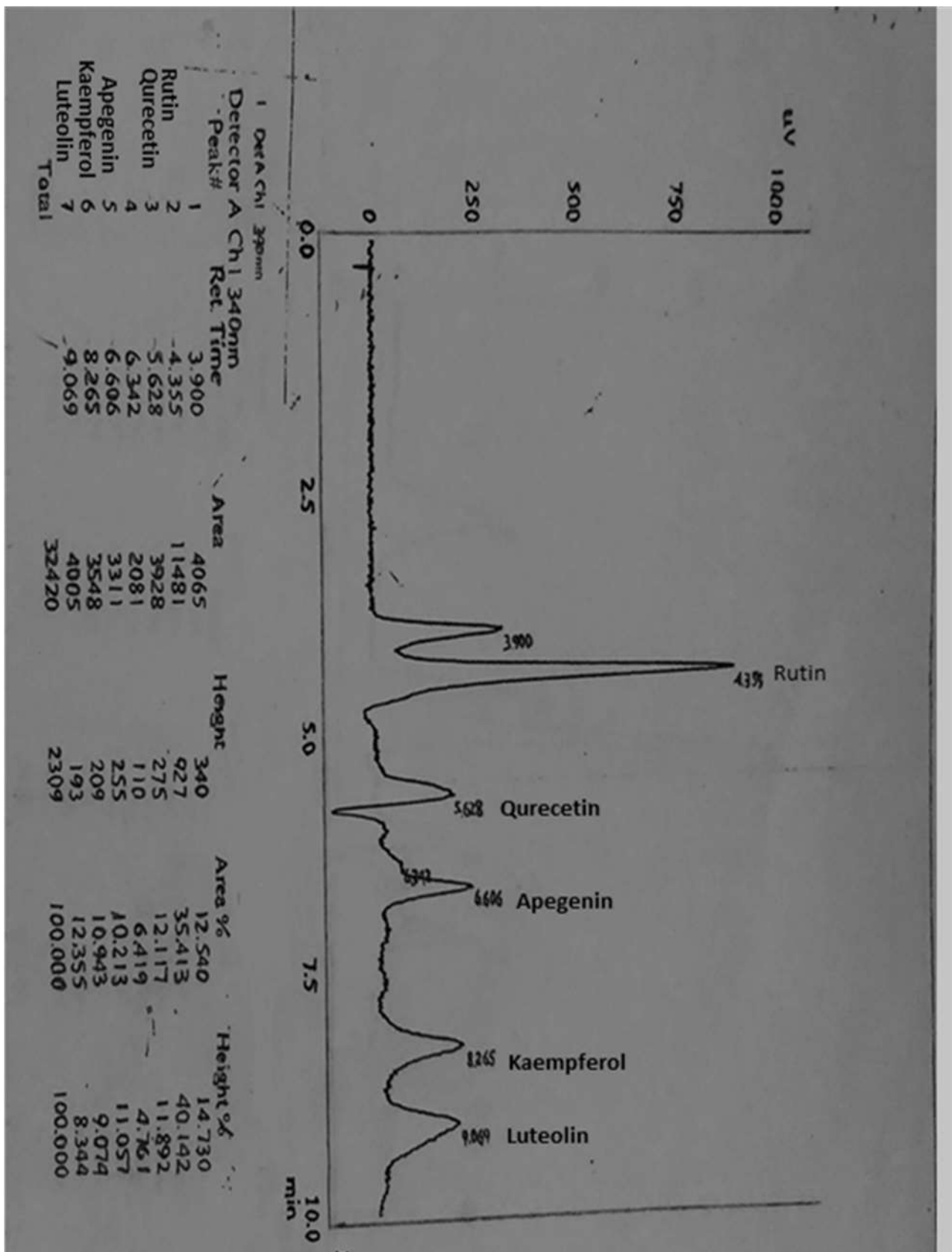
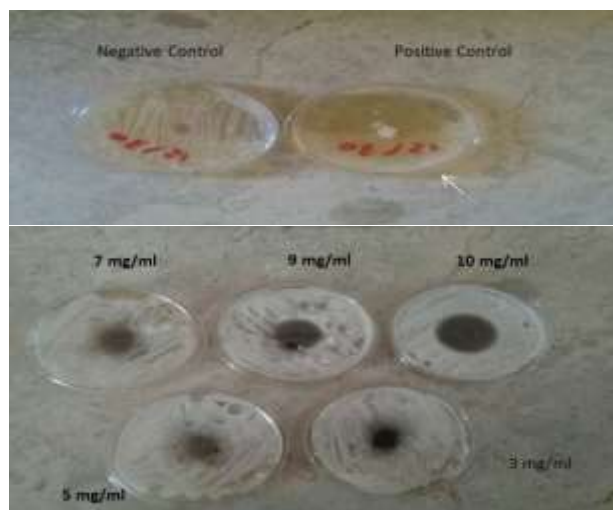


Figure 3. HPLC Chromatogram of the standard flavonoids

**Antifungal activity**

The evaluation of inhibitory effect for flavonoid of *M. communis* against *T. mentagrophytes* revealed high inhibitory effect with concentration 10mg/ml of flavonoid with 36.67 mm; inhibition zone And The minimum

inhibitory concentration (MIC) values of *M. communis* flavonoid against *T. mentagrophytes* was, 3 mg/ml Figure4.



**Figure 4.** inhibitory effect of *Myrtus communis* flavonoids on the *T.mentagrophyt* grown on (SDA) medium after 7 days of incubation at 28 C°

**Table 2.** Minimum inhibitory concentration (MIC) of *M. communis* flavonoid (well diffusion technique ) after 7 days against *T. mentagrophytes*.

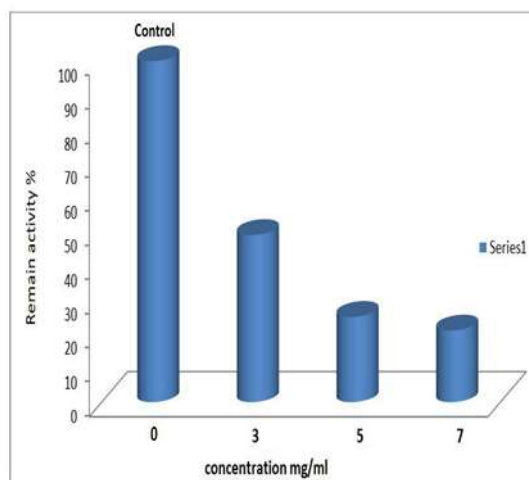
| Different conc.(mg/ml) of <i>M.communis</i> flavonoid and inhibition zone in mm |    |    |    |    |         |                          |
|---------------------------------------------------------------------------------|----|----|----|----|---------|--------------------------|
| 3                                                                               | 5  | 7  | 9  | 10 | control | klorancazole (75mg vol.) |
| 24                                                                              | 27 | 31 | 35 | 37 | 0       | 5.6 mm                   |
| positive control klorancazole                                                   |    |    |    |    |         |                          |

The main groups of phenolic compounds are flavonoids, phenolic acids, tannins, stilbenes and lignans (22). Flavonoids are probably the most important group of phenolic compounds. Flavonoids are known to be synthesized by plants in response to microbial infection, Antibacterial flavonoids might be having multiple cellular targets, rather than one specific site of action. One of their molecular actions is to form complex with proteins through nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation. Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesions, enzymes, cell envelope transport proteins, and so forth. Lipophilic flavonoids may also disrupt microbial membranes (29,31). As general , the differences of inhibitory effect of antifungal agents due to the natural of fungi itself include the differences of the structure in cellular membranes and its thickness ,the size of fungal cells and the duration of the growth (24). Studies demonstrated the effectiveness of flavonoid against dermatophytes, Bhadauria and Kumar (4) when they found that flavonoids

of *Cymbopogon martinii* leaves showed inhibition zones of 28 and 38 mm for *T. mentagrophytes* and *M. gypseum*. Recently, In 2019 flavonoid were elevated from *Salacia senegalensis* (Lam.)as antidermatophytic (11).

**Effect of flavonoid on keratinase activity**

The inhibitory effect of three different concentration 3, 5 and7 mg/ml of *M. communis* flavonoid on keratinase activity revealed that There was an increase in the inhibition the enzyme activity as the increase of concentrations of *M. communis* flavonoids ,the lower remain activity of the enzyme was 21% at 7 mg/ml of *M. communis* flavonoid (Figure5).



**Figure5.** inhibitory effect of *Myrtus communis* flavonoid on keratinase activity

keratinase have key roles in fungal invasion and pathogenesis in human and animal dermatophytosis (27,34). Enzyme inhibitors prevent enzymes from their catalytic function by interfering with any step in the catalytic cycle (32,33). They are low molecular weight compounds that in small quantity can reduce or completely inhibit the enzyme activity (10,12). Many researches studying the inhibition effect of plants crude extracts or plants compounds on keratinase activity ,Guerrero (13) evaluated the ability of 17 flavonoids belonging to five structural subtypes to inhibit angiotensin-converting enzyme (ACE) and showed that the highest activity was obtained for luteolin. And (37) demonstrated that quercetin is a member of the flavonoids inhibit trypsin-like serine proteases at micromolar potencies. *Trichophyton mentagrophytes* was the most common isolate obtained in present work This study found out that the inhibitory effect of the flavonoid extract from *M. communis*



against *T. mentagrophytes* and keratinase activity was 10, 5 mg/ml respectively. Further studies should be carried out other dermatophyte isolates included in the study.

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