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ORIGINAL ARTICLE

# Comparative Evaluation of Alkaloids Extraction Methods from the Root Bark of *Punica granatum* Linn

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**ABSTRACT**
*A comparative study was carried out to evaluate alkaloid antibacterial activity which was extracted from the root bark Punica granatum L. by liquid membrane techniques (SA) and organic solvent traditional techniques (SB) . The screening of the antimicrobial activity was conducted by agar well diffusion method against Staphylococcus aureus, Enterobacter cloacae, Escherichia coli , Klebsiella pneumoniae , and Proteus mirabilis at three concentration levels (5,10 and 15 mg/ml) . Alkaloid extracts were analyzed by a high performance liquid chromatography (HPLC) method . Among the tested extractions, SB showed the highest antibacterial activity against all five bacterial strains, especially at 15 mg/ml concentration . However, all the B type solutions concentrations were significantly affected against tested bacteria. The most susceptible bacteria to SA were E. coli , followed by Proteus mirabilis while the most resistant bacteria were Enterobacter cloacae and Klebsiella pneumoniae , followed by Staphylococcus aureus . Bioautography showed that the antimicrobial activity was probably due to Pelletierine compounds.*
*Keywords: antibacterial activity, Punica granatum Linn. , Liquid Membrane Extraction, Organic Solvent Extraction*

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## INTRODUCTION

Plants have been used as medicine since time immemorial. Many of the natural products in plants of medicinal value offer us new sources of drugs which have been used effectively in traditional medicine. There in an increased consciousness regionally and globally in the production and use of plant with healing property [1]. The extraction and characterization of active compounds from medicinal plant have resulted in the discovery of new drugs with high therapeutic values [2]. In all these operations , methods are needed for the separation, purification and identification of the many different constituents present in plants. Thus, advances in our understanding of photochemistry are directly related to the successful exploitation of known techniques, and the continuing development of new techniques to solve outstanding problems as they appear [3].

Alkaloids, widely existing in natural plants, are compounds containing basic nitrogen atoms. Most of alkaloids are pharmacologically active ingredients in many medicinal plants due to their significant physiological activity. Many alkaloids can be extracted from natural plant materials and purified by modern separation techniques [4] . Pelletierine C8H15NO, is a liquid alkaloid obtained from the root bark of Punica granatum Linn. It is an anthelmintic and amoeboid . Pelletierine triggers, like strychnine, a raised stimulant reflex, which can escalate to tetanus , and it is effective against diverse tapeworms, ring worms and nematodes [5] . More recent studies showed that the simple extract have efficacy against viruses including such as *Herpes simplex* [6]and human immunodeficiency virus (HIV) and tumors [7] . Pelletierine is said to occur in the bark of Punica granatum L. together with pseudo pelletierine, isopelletierine and methyl pelletierine. It is evidents that pelletierine has anthelmintic properties and that the anthelmintic activity of the mixture of alkaloids from Punica granatum is mainly (or considerable extent) due to the presence of pelletierine in these mixtures [8].

Solvent organic extracts contain a mixture of secondary metabolites including alkaloids, flavonoids, terpenoids, and other phenolic compounds; these molecules are associated with the defense mechanisms of plants by their repellent or attractive properties, protection against biotic and abiotic stresses, and maintenance of structural integrity of plants [9].

The liquid membrane extraction was introduced as an alternative separation technique to the liquid-liquid extraction and to the separation by means of solid polymeric membrane. This property of membranes makes them useful in the textile and food industries, in hydrometallurgy, medicine, biotechnology, environmental protection, in the separation of hydrocarbons and gases, and in the concentration and separation of amino acids, metal ions and other mixtures and suspensions [9 ,10] .
The aim of the present work was to evaluate the antibacterial activity of Alkaloids extraction methods from root bark of *Punica granatum* L. by using liquid membrane and organic solvent traditional techniques.

## MATERIALS AND METHODS

### Plant extract preparation

### 1. Leaching

Five grams of *Punica granatum* L. roots were milled to fine powder then leached by 250 ml of buffer solution of ($NH_3$-($NH_4$)$2SO_4$) and adjusted to appropriate pH . This solution was shaken for half an hour and filtered to obtain the feed solution.

### 2. Extraction

### A . liquid membrane industrial techniques (SA) .

Bulk liquid membrane process for the recovery of medicinal compounds from dilute ammoniacal leach solutions has been studied in this work . Applying pertraction in a rotating film contactor (RFC) the alkaloid was successfully recovered from model solution of Pelletierine, as well as from native aqueous extracts obtained from the *Punica granatum* L. roots.
The solution from the above step was poured into the rotating film contactor ( RFC) using glass funnel, the acceptor solution was adjusted to appropriate pH using few drops of sulfuric acid and placed into the second compartment, the remainder volume was filled with the liquid membrane.

### Reagent and Analytical Methods Used

Various reagents were used in this work as liquid membrane, n-decane and n-nonane (99% BDH), n-hexane (95% ALDRICH) and methyl cyclohexane (95% HOPKIN & WILLIAMS). Ammonia (25% CHEM-SUPPLY) and sulfuric acid (98% GCC) were used to adjust the acidity of the aqueous solutions. The concentration of pelletierine in the strip solution was measured by UV-spectrophotometer SP-3000 (OPTIMA INC) at wave length λ=254 nm. The pH values of the aqueous solutions were measured by means of a laboratory pH meter (CRISON, MM40).

### Experimental Equipment

The studies of pelletierine pertraction were carried out in a laboratory rotating film contactor (RFC) made from Perspex (poly methyl methacrylate). As shown in Fig.1,The lower part of contactor is divided, , into four compartments: two for the feed and two for the acceptor solution. Compartments containing the same aqueous solution are interconnected. The organic membrane liquid occupies the common upper part of the contactor.

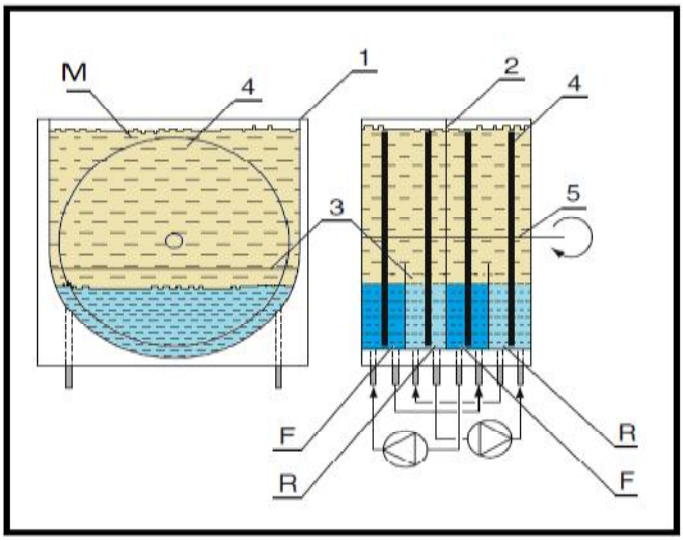



**Fig.1:** Experimental apparatus contactor: (1) body of rotating film contactor, (2) stage wall (3) feed/stripping solution separating walls, (4) rotating disks, and (5) common shaft.

Four discs, 1 mm thick and 18 cm in diameter, mounted vertically on a common shaft, rotated in each compartment, providing continuous renewal of the aqueous films, covering the discs, as well as the stirring of all three liquids. The lower part of each disc (up to one-third of the disc diameter) is immersed in the corresponding aqueous solution while the larger upper part is immersed in the organic membrane liquid, as shown in Fig.1( the aqueous solutions from mobile liquid films on the corresponding disc surfaces, which contact with the organic membrane) .The two stages connected in a way permitting co-, counter or batch operation modes. To homogenize the aqueous solutions and to provide samples from each solution, both liquids were re-circulated by means of two peristaltic pumps Watson-Marlow Limited (Falmouth Cornwall England).

For the pertraction studies, the following three-liquid-phase system was used:

Feed (donor) solution (F): 250 ml aqueous solution of ammonia, containing 1.13 mmol/liter of Pelletierine C8H15NO;

Membrane solution (M): 500 ml : n-decane, n-nonane, n-hexane and methyl cyclohexane; Stripping solution (S): 250 ml aqueous solution of sulfuric acid.

Experimental Procedure

In the beginning (5.0) grams of Punica granatum L. roots were milled to fine powder and leached by 250 ml of buffer solution of (NH3-(NH4)2SO4) and adjusted to appropriate pH . This solution was shaken for half an hour and filtered to obtain the feed solution The solution from the above step was poured into the RFC using glass funnel, the acceptor solution was adjusted to appropriate pH using few drops of sulfuric acid and placed into the second compartment, while the remainder volume was filled with the liquid membrane [11] .

**B. Organic solvent traditional laboratory techniques (SB)**

Crude Alkaloids were extracted according to [12] . 100 gm of plant powder was mixed with 350 ml methanol : D.W. mixture in a ratio of 1:4 in electrical blender for 5 minutes. The solution was filtered through muslin cloth , then through Buchner funnel under reduced pressure by using a filter paper (Whattman No.1).

The supernatant was evaporated at 45ºC in a rotary evaporator, drops of 2% Sulphuric acid were added to make pH=1-2 , then transferred to a separation funnel and extracted with chloroform three times. The solution was separated into two layers, the lower layer is chloroform layer which was neglected, while the upper layer is the aqueous layer . Concentrated ammonium hydroxide was added to this layer to make pH=9, then the solution was extracted in a separation funnel with chloroform : methanol mixture in ratio of 3:1 twice, and one time with chloroform alone. The solution was separated into two layers, the lower layer, chloroform layer or chloroform : methanol layer, then it was evaporated in a rotary evaporator . The upper layer , the aqueous layer, was evaporated in rotary evaporator, the residue was extracted with methanol, then the extract was kept in refrigerator .

**3. Antibacterial activity assay**

The assay for antibacterial activity of each plant part extract was tested by agar well diffusion method [13] . Bacterial suspensions were cultured in peptone water for 6-8h at 37 Cº and 0.2ml of this culture was spread over Mueller – Hinton agar in Petri dishes. Wells (8mm diameter) were cut in agar plates and were filled with 0.1ml of 15% plants extracts. The plates inoculated with Xanthomonas campestris pv. campestris were incubated at 30±1ºC. The resulting zone of inhibition was measured after 24 h. Each combination of isolates and antimicrobial agent was repeated three times. The isolate which showed a clear zone of inhibition more than 12mm including the 8mm well size was considered sensitive and those with less than 12mm as resistant.

**4- Separation of alkaloid in pomegranate root by HPLC**

The analysis of the chemical composition was made by High performance liquid chromatography (HPLC). HPLC consists from a mobile phase which is polar and consists of a mixture of solvents such as water and acetonitrile, while the stationary phase comprises of a column which is usually stainless steel and packed with silica particles, a sample of 50µl was injected into the mobile phase using a procedure outlined by Hartley and Buchan and it passes along the stationary phase, the time taken for a sample to pass through the system is recorded, this represent the retention time and is considered as one of the characteristic used to identify the compound. All the compounds were separated and identified using HPLC with separation conditions Lichrospher C18 , 3µm, particle size, 50× 4.6 mm internal diameter of the column, detection U.V. set at 254 nm, flow rate 1.2 ml/min. and 30 C° temperature, acetonitrile: 0.01M sodium dodecyl sulphate (60:40, V/V).

The sequences of the eluted material of the standard were as follows, in which each standard was 25 µg/ml.

**Table (1)** HPLC analysis for standard Pelletierine.

| Seq. | Subjects | Retention time (min.) | Area | Concentration (µg/ml) |
|---|---|---|---|---|
| A. | Pelletierine(punicine) | 2.95 | 36437 | 25 µg/ml each |

The area under a peak was used for calculating the concentration of a sample as shown in the following formula:

Concentration of sample (µg/ml) = [[Area of the sample/Area of the standard]] × Standard Conc. × Dilution factor

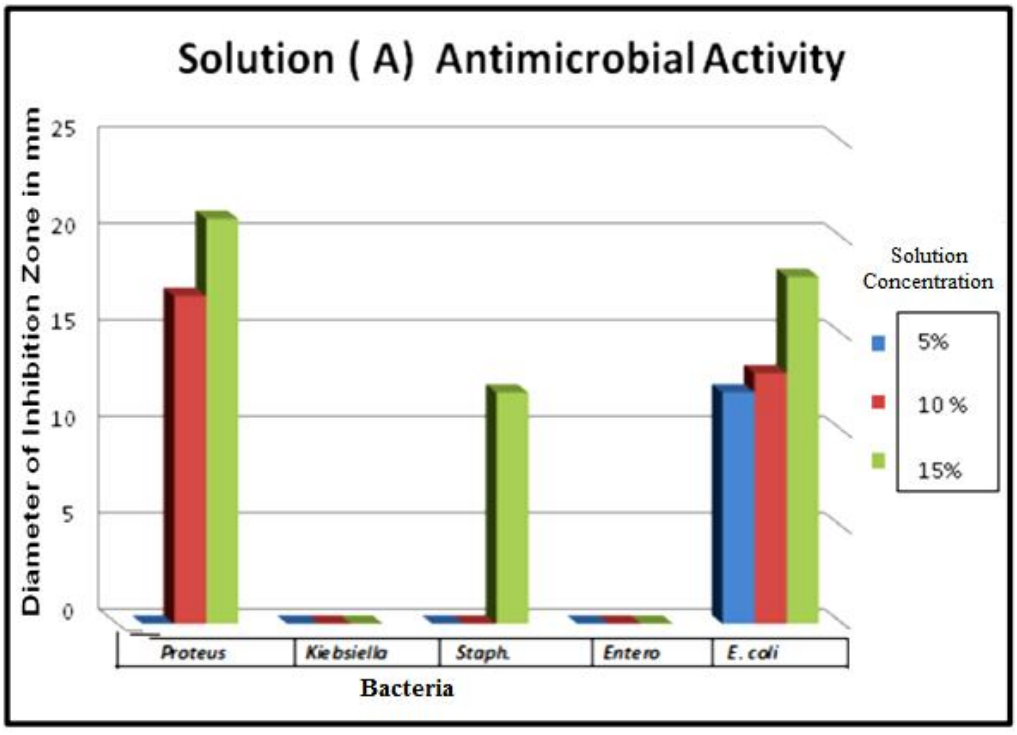
**Statistical analysis**

Complete Randomized Design (C.R.D.) was used as an experimental design. Data were analyzed by using the statistical analysis system- SAS (2001) to study the effect of different factors on the diameters of inhibition zones, Least significant difference (LSD) was used to compare the significant differences between means at P≤ 0.05.

## RESULTS

The Effect of (SA) & (SB) extracts on the bacterial cultures screening results are presented in Figures 2 and 3.

In Figure 2, The most susceptible bacteria to (SA) were E. coli , followed by Proteus mirabilis while the most resistant bacteria were *Enterobacter cloacae* and *Klebsiella pneumoniae* , followed by *Staphylococcus aureus* . Although the most concentration active was 15% but not for all bacteria especially Poteus mirabilis (21mm).

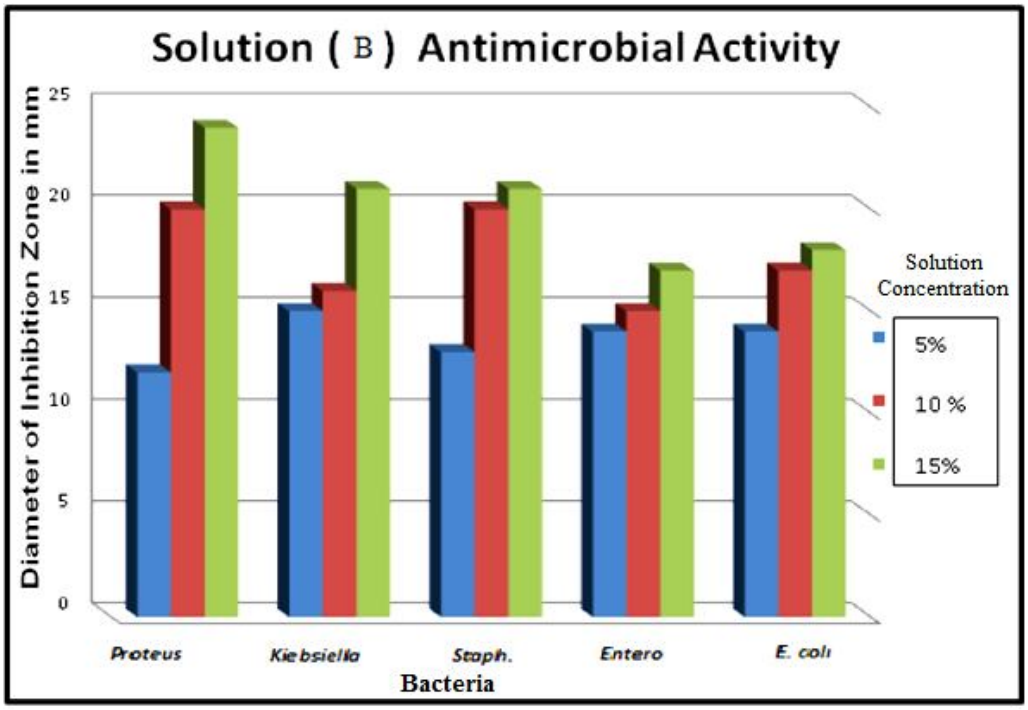



L.S.D 5% Between Con. 1.298
between bacteria 1.675
Between Con. and bacteria 2.902

**Fig. 2**. Effect of(SA) extracts on the bacterial cultures.

However plant extracts in organic solvent (methanol) provided more consistent antimicrobial activity compared to those extracted in liquid membrane techniques . All the (SB) concentrations were

significantly affected against these Bacteria , and the compounds showed significant activity at the given concentration levels , especially at 15 mg/ml concentration which was shown to be most active on Proteus mirabilis ( 23mm) and least on Enterobacter cloacae ( 16 mm)(figure 3).




L.S.D 5% Between Con. 1.298
between bacteria 1.675
Between Con. and bacteria 2.902

**Figure 3:** Effect of(SB) extracts on the bacterial cultures

**CHEMICAL CONSTITUENTS**

The results of the phytochemical screening revealed that (HPLC) shows the relative concentrations of various compounds getting eluted as a function of retention time (Figure 4 , 5 and 6) . The heights of the peak indicate the relative concentrations of the components present in the plant (Figure 4). Results of HPLC of the (SA) referred to the presence of alkaloids Pelletierine only (Figure 5) ,while of the (SB) referred to the presence of alkaloids Pelletierine with anther compounds, these compound could be (Methylpelletierine, Methyl-iso Pelletierine , Isopelletierine.) (Wibaut and Hollstein, 1956) (Figure 6).

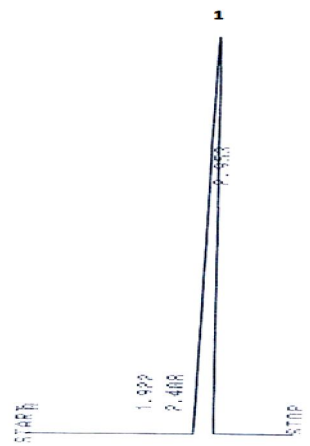



Fig. 4. HPLC scanning for standard Pelletierine

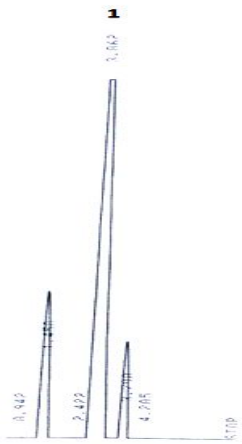



Fig. 5 . HPLC scanning for alkaloids extracted by RFC (S A).

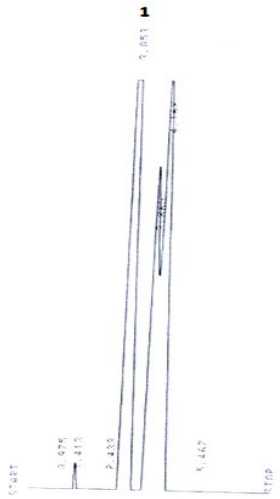


Fig 6. HPLC scanning for alkaloids extracted by organic solvent techniques (SB).

## DISCUSSION

The results obtained in this study indicate a considerable difference in antimicrobial activity between extracts obtained with liquid membrane techniques (RFC) method and those obtained by organic solvent traditional techniques (methanol) method, the diethyl ether extracts (SB) being more active than the other extracts (SA). This is due to the fact that Alkaloids compounds from plant material is largely dependent on the type of solvent and the method used in the extraction procedure; besides the plant extracts in organic solvent (methanol) provided more consistent antimicrobial activity compared to those extracted in RFC techniques . Moreover the use of liquid membranes presents an attractive approach to produce valuable products of high quality but less powerful yet more selective than in the case of classical solvent extraction . However ,These observations can be rationalized in terms of the polarity of the compounds being extracted by solvent, in addition to their intrinsic bioactivity, by their ability to dissolve or diffuse in the different media used in the assay. Bioautography showed that the antimicrobial activity was probably due to Pelletierine compounds. However, by comparing antibacterial activity, it was observed that the Alkaloids methanol extract (SB) was more active than that extracted by RFC (SA) fractions; which indicate that the activity may be due to the combined effects of the referred to present of Pelletierine compunction such as Methylpelletierine, Methyl-iso Pelletierine , Isopelletierine [8]. More recent studies showed that the Pelletierine from *Punica granatum* L. by simple extract from roots has an efficacy against the virulent intestinal bacteria *salmonella typhi* [14] . It has been shown to be a good alternative to the synthetic chemical substances to prevent the growth of Staphylococci and E.coli [15].

## CONCLUSIONS

The use of liquid membranes presents an attractive approach to produce valuable products of high quality with less powerful but more selective than in the case of classical solvent extraction.Thus such an end, It may be worth looking deep into chemical constituents of different parts of Pomegranate and their activity profiles either individually or in combination.

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