The use of the water extract of *Rosa spp* petals as a bacterial growth medium

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الخلاصة:

تم تحضير مستخلص مائي من الاوراق التويجية لنبات ورد الجوري ***Rosa spp.*** تحت ظروف معقمة، ثم استخدم لأول مرة كوسط زرعي تجريبي لتنمية البكتريا:

***Pseudomonas aeruginosa*, *Proteus vulgaris*, *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pneumoniae and Klebsiella pneumonia*.**

استُخدِمَ هذا الوسط التجريبي كبديل عن الاوساط الزرعية التقليدية (وسط الأكار المغذي، وسط أكار ماكونكي و وسط أكار الدم) التي تستعمل لتنمية هذه الاجناس في المختبر.

أظهرت جميع أنواع هذه البكتريا نمواً فعالاً بعد (24) ساعة من الحضن عند استعمال الوسط كما هو بشكله السائل. كما استعمل المستخلص في اغناء الاكار agar–agar وزرعت عليه نفس انواع البكتريا وأظهرت نمواً ملفتاً للانتباه. تقترح النتائج ان هذا المستخلص يوفر وسطاً زرعياً مناسباً لتنمية هذه البكتريا وممكن الاستعاضة به عن الاوساط الزرعية الاصلية التي تستعمل لتنمية هذه البكتريا في المختبر. كما يوفر هذا الوسط بيئة مغذية غنية و مهمة لا تقل اهمية عن البيئات المغذية التي توفرها الاوساط الاخرى.

Abstract:

In a pioneer study, simple water extract for the red petals of *Rosa spp.* was prepared under sterile conditions, then used for the first time as experimental bacterial culture medium for the growth of the bacteria: ***Pseudomonas aeruginosa*, *Proteus vulgaris*, *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pneumoniae and Klebsiella pneumoniae***; the medium was used as alternative culture medium for the routine culture media (Nutrient agar, MacConkey agar and blood agar) that used for the growth of these genera in the laboratories.

All the genera showed active growth after 24 hours when it used directly as a liquid culture medium. The extract was used also to enrich the agar-agar and cultivated with the same bacteria; it showed a noticeable growth. The results suggest that this extract is a suitable culture medium; it could be used instead of the routine culture media that used in the cultivation of these bacteria in the

laboratories. It also represents important, rich nutritional medium as those that is used in the routine laboratory work.

Introduction:

For any bacterium to be propagated for any purpose it is necessary to provide the appropriate biochemical and biophysical environment. The food base that supports the growth of an organism is called culture medium; the biochemical (nutritional) environment is made available in this culture medium[1-4].

A growth medium is a mixture of nutrients, moisture and other chemicals that bacteria need for growth in a laboratory environment. Media can be solid, such as Jell-O-like agar that is poured into the bottom half of a Petri dish, or media can be liquid to allow for bacterial growth in test tubes [1-5].

The food base depending upon the special needs of particular bacteria (as well as particular investigators), so that a large variety and types of culture media have been developed with different purposes and uses [1,2]. These include sources of organic carbon, nitrogen, phosphorus, sulfur and metal ions including iron[6]. Culture media are employed in the isolation and maintenance of pure cultures of bacteria and are also used for identification of bacteria according to their biochemical and physiological properties [1,3,4].

The manner in which bacteria are cultivated, and the purpose of culture media, varies widely. Liquid media are used for growth of pure batch cultures, it include media may be made from animal tissue and fluids, e.g., nutrient broth, serum broth, carbohydrate broths, milk, blood, nitrate peptone solution, Dunham's solution; or from vegetable tissue such as malt extract (germinated barley), beer wort, yeast extract, hay infusion, natural fruit juices, wines (fermented fruit juices) [3,4]. Solidified media are used widely for the isolation of pure cultures, for estimating viable bacterial populations, and a variety of other purposes; the usual gelling agent for solid or semisolid medium is agar, a hydrocolloid derived from red algae. Agar is used because of its unique physical properties (it melts at $100^{o}C$ and remains liquid until cooled to $40^{o}C$, the temperature at which it gels) and because it cannot be metabolized by most bacteria. Hence as a medium component it is relatively inert; it simply holds (gels) nutrients that are in aqueous solution [1,3,4].

Every organism must find in its environment all of the substances required for energy generation and cellular biosynthesis. The chemicals and elements of this environment that are utilized for bacterial growth are referred to as nutrients or nutritional requirements. Many bacteria can be grown in the laboratory in culture media which are designed to provide all the essential nutrients in solution for bacterial growth [1,3-5].

Basically, the culture media are of three types: natural, synthetic and a complex (undefined) media. Natural medium is that which contains the natural products such as, for example diluted blood, urine, milk, vegetable juices,

peptone or animal cells/tissues/organs. In such medium the exact chemical composition is not known. A synthetic medium is one chemically-defined in which the exact chemical composition and concentration is known. A complex (undefined) medium is one in which the exact chemical constitution of the medium is not known [1,5,7].

Other concepts employed in the construction of culture media are the principles of selection and enrichment. A selective medium is one which has a component(s) added to it which will inhibit or prevent the growth of certain types or species of bacteria and/or promote the growth of desired species. A culture medium may also be a differential medium if it allows the investigator to distinguish between different types of bacteria based on some observable trait in their pattern of growth on the medium; MacConkeys & Mannitol Salt Agars in addition to being selective, they are also differential[1,3-5,8-11]. An enrichment medium employs a slightly different twist. It contains some component that permits the growth of specific types or species of bacteria, usually because they alone can utilize the component from their environment. However, an enrichment medium may have selective features [1,3,5,8,10]. Blood agar is an enriched medium that provides an extra rich nutrient environment for microbes. It contains 5% sheep blood; therefore (BAP) is not a selective growth medium, since it supports the growth of a wide range of organisms[3-5,8,11,12]. Nutrient agar is used for the cultivation of bacteria and for the enumeration of organisms in water, sewage, feces and other materials. It is used in the laboratory for the cultivation and maintenance of nonfastidious species [3,5,7,11,12].

In a pioneer study, a simple water extract for the red petals of *Rosa spp.* was prepared under sterile conditions, then used as an experimental culture medium to examine its ability to produce a suitable source of nutritional requirements for many types of (Gram+) and (Gram-) bacteria as a substitute culture medium for the routine bacterial culture media (MacConkey agar, blood agar and nutrient agar) that used for the growth of these types in the laboratories; the types of used bacteria were: ***Pseudomonas aeruginosa*, *Proteus vulgaris*, *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pneumoniae and Klebsiella pneumoniae***. In the first experiment the extract used directly as a liquid culture medium; in the second experiment the extract used to enrich the (agar-agar) and it used as a solid culture medium. It could be considerd this medium (the extract) from the natural type of media because it is from natural source (vegetable juice), its exact chemical composition not known although the presence of some components is expected; such components are water, protein, lipids, aromatic oil, polysaccharides and salts [13]. The availability of the suitable nutrients and the fitting of medium for the growth were investigated by the bacterial growth in the liquid and enriched solidified medium; the growth of these bacteria compared with their growth in the original media. The aim of this study is to find an alternative medium characterized by cheap cost and simple

preparation could be used instead of the routinely prepared media that is used in the laboratories to grow these types of bacteria.

Materials and Methods:

The preparation of the experimental medium (the extract):

The preparation of the extract done according to Al-Succary *et al* [13] with minor modification from Al-Azzauy[14]; where the red petals of *Rosa spp*. were collected and washed carefully with tap water to remove the dust and other materials. Then (250)g of these red petals were putted in (1)L beaker and (250)ml of distilled water were poured in the beaker, the mixture were heated for (10)minutes in (100)˚C. The extract refines by the refinery then cooled to room temperature, and then it is filtered by Whatman filter paper, the pH adjusted at (7.4). After then the extract were sterilized by millipore filter (0.22)µ, putted in sterile bottles and preserved in (4)˚C to be ready for use then.

The use of the extract in the bacterial growth:

1- The extract as liquid growth medium

The extract were dispensed into many sterile universal tubes (2 ml in each one) and used as a liquid growth medium. The bacteria were selected from (24) hrs. incubated bacterial suspensions, turbidity was visually adjusted to that of (0.5) McFarland turbidity standard (1.5×10^8) Colony Forming Unit (CFU)/ml. The extract inoculated with (0.1)ml of the bacterial suspensions of ***P. aeruginosa*, *P. vulgaris*, *S. aureus*, *E. coli*, *S. pneumoniae*, *and K. pneumoniae*** respectively, these types of bacteria were collected from patients and all the isolates were stained with Gram stain and diagnosed by the biochemical tests: urea, Simmon citrate, TSI, Indol and motility for (Gram-) bacteria (***P. aeruginosa, P. vulgaris, E. coli*** and ***K. pneumoniae***), and the tests: catalase, coagulase for (Gram+) bacteria (***S. aureus, S. pneumoniae***) and mannitol salt agar for ***S. aureus***[15]. After (24) hrs of incubation at (37)˚C, the bacterial growth, color of the medium and the pH of the medium were investigated. The intensity of growth measured by the turbidity, color and pH change. The viability of the bacteria investigated every day after the preliminary period of incubation (the 24 hrs.) by making subcultures, the purpose of this step were to estimate the ability of this medium in the maintenance of the bacteria.

2- The solidified growth medium (the extract as enrichment substance to the agar-agar)

After the preparation of agar-agar (Biolife company) it sterilized by autoclave then left to cool for (40)˚C and enriched with the prepared extract; this is done by the dilution of (15)gm of agar-agar in (200)ml of distilled water followed by adding (800)ml of the extract. After then the medium poured into sterile Petri dishes then cultivated by spreading of (0.1)ml of bacterial suspensions(0.5) McFarland turbidity standard (1.5×10^8) CFU)/ml by the loop from the same types of bacteria mentioned above (***P. aeruginosa*, *P. vulgaris*, *S. aureus*, *E. coli*, *S. pneumoniae*, *and. pneumoniae***) respectively. After

incubation at (37)˚C for (24)hrs. the bacterial growth, the color of the medium and the colonies were investigated. The viability of the bacteria investigated every day after the preliminary period of incubation (the 24 hrs.) by making subcultures as mentioned above.

3- Examination of the growth cells (in the liquid medium) and the colonies (in the solid medium) using Gram stain:

After the incubation period and the appearance of the growth in the both experimental media (liquid medium and enriched solidified medium), the biochemical tests mentioned above were repeated for all the types of the used bacteria; as well as many samples were collected from each type of these experimental media for microscopic examination and stained with Gram stain to ensure that this growth was from the certain selected isolate and not because of any contamination.

Results:

After the incubation period, each type of media (the liquid and enriched solidified media) showed noticeable bacterial growth. The color and pH were changed in the liquid medium; they are synchronized with the average bacterial growth which is detected by the turbidity of the medium. All the used types of bacteria still viable in this medium for (7) days.

The color of the medium converted from red to yellow in the medium inoculated with ***S. aureus*, *S. pneumoniae*, *and K. pneumoniae***, while it is converted to dark yellow, dark brown and light brown in the medium inoculated with ***E. coli, P. aeruginosa*** and ***P. vulgaris*** respectively, this is very explicit in the figures (2) and (3). The pH of the liquid medium was increased from (7.4) to (6.3) after the growth of ***S. aureus*, *E. coli*, *S. pneumoniae*, *and K. pneumoniae*** in the medium; while increased to (5.9) in the medium inoculated with ***P. aeruginosa*, *P. vulgaris***. (Table-1) summarized these results.

The solidified enriched medium showed considerable bacterial growth but the color of medium not changed with growth. The color of the bacterial colonies was mostly transparent in ***S. aureus, E. coli*** and ***S. pneumoniae***, while the colonies of ***P. aeruginosa*** were brown to grayish, and were grayish in ***P. vulgaris*** and ***K. pneumoniae***, (Table -2). All the used types of bacteria still viable in this medium for (4-5) days.

After the staining of the slides which prepared from the growing isolates in both the liquid and solid media with Gram stain, the microscopic examination showed (Gram-) rods present as single bacteria or in pairs or in short chains in the slides prepared from the cultures of ***P. aeruginosa***; in the slides prepared from the cultures of ***P. vulgaris*** there were (Gram-) rods, while in the slides prepared from ***S. aureus*** cultures there were (Gram+) cocci single, pairs, tetrads and chains arranged in irregular clusters. The microscopic examination of ***E. coli*** cultures showed short (Gram-) rods; the slides of ***S. pneumoniae*** showed

(Gram+) single cocci or pairs and the slides of ***K. pneumoniae*** showed (Gram-) rods with large capsule.

Type of Bacteria	**The color of liquid medium (the extract)**		**The pH of the liquid medium (the extract)**	
	Before growth	**After growth**	**Before growth**	**After growth**
Pseudomonas aeruginosa	Red	Dark brown	7.4	5.9
Proteus vulgaris	Red	light brown	7.4	5.9
Staphylococcus aureus	Red	Yellow	7.4	6.3
Escherichia coli	Red	Dark Yellow	7.4	6.3
Streptococcus pneumonia	Red	Yellow	7.4	6.3
Klebsiella pneumoniae	Red	Yellow	7.4	6.3

Table-1: The color and pH changes in the liquid medium (extract) before and after inoculation with different types of bacteria.

Bacteria	**Color of colonies in**			
	solidified medium (the extract)	**MacConkey agar**	**Blood agar**	**Nutrient agar**
Pseudomonas aeruginosa	Brown to Grayish	-	-	Green
Proteus vulgaris	Grayish	-	-	White
Staphylococcus aureus	transparent	-	Grayish	-
Escherichia coli	transparent	Pink	-	-
Streptococcus pneumoniae	transparent	-	Transluc ent	-
Klebsiella pneumoniae	Grayish	-	-	White

Table-2: The color of the colonies in the solid medium (the solidified extract), MacConkey agar, blood agar and nutrient agar.

* The sign (-) mean that this medium was not used for the growth of this type of bacteria.

Figure-1: The liquid culture medium (the extract)

Figure-2: The liquid medium after the cultivation with before ultivation.
(from left side): *S. aureus*, *S.pneumoniae*, *K. pneumoniae*, *P. aeruginosa and P. vulgaris*.

Figure-3: liquid medium after cultivation with *E. coli*.

Figure-4: Solid enriched medium without cultivation.

Figure-5: Cultivated solid enriched medium.
Right: *P.vulgaris*.
Left: *K. pneumoniae*.

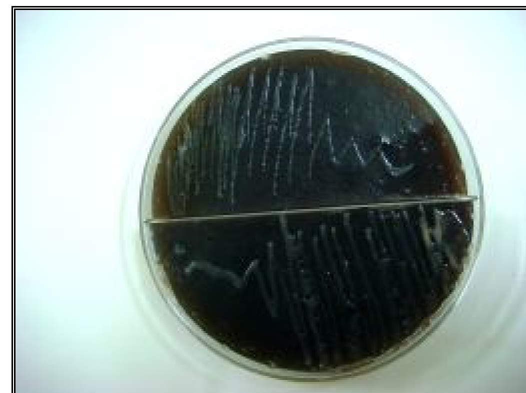

Figure-6: Cultivated solid enriched medium.
Up: *P. vulgaris*.
Down: *P. aeruginosa*.

Figure-7: Cultivated solid enriched medium.
Right: ***E. coli*****, left:** ***S. pneumoniae*****.**

Figure-8: Cultivated solid enriched medium.
Up: ***S. aureus*****, down:** ***S. pneumoniae***

Discussion:

The results suggest that the liquid medium (the extract) is a good substitute for the nutrient broth in the cultivation and maintenance of ***P. aeruginosa***, ***P. vulgaris***, ***S. aureus***, ***E. coli***, ***S. pneumoniae***, and ***K. pneumoniae***. This medium represents a suitable medium for the growth of all the used types of bacteria although it is from plant origin, this fittings very clear from the intensity of the growth that obtained from the inoculation of the medium with the above types of bacteria which measured by the turbidity, the color and pH change as it seen in (figures 2 and 3); moreover, the medium maintained all the used types of bacteria for at least (7) days with full viability. This is may interpreting as that this medium provides these types of bacteria with all the essential requirements for their growth.

This experimental medium has a red color because of the use of the red petals of ***Rosa spp***. in the extraction; the color resulting from the natural stain that present in the cell juice of the petals' cells (Anthocyanin) which become free in the extract solution after the destruction of the cells by the heat during the preparation of the extract figure(1). This color changed after the bacterial growth in the medium which resulting in acidic metabolic products as a result of the fermentation in some of the extract's components by the bacteria; these products contributed in the change of the pH, and because the Anthocyanin color changing with pH, then the stain's color converted since the pH of the medium changed. This change gave a good indicator for the presence of the growth, as well as to the turbidity, from one hand and used as an evidence for the pH change (which then approved by the litmus paper) from the other hand; this character reduced the need for adding any dye to the prepared medium (Table-).

The solidified growth medium produced a suitable environment for the bacterial growth and met the nutritional requirements for the growth of these types of bacteria. The success of this medium in its job approved by the bacterial growth that obtained after the cultivation of the bacteria as it appears in the

figures (5, 6, 7 and 8). The growth of (Gram+) bacteria was less intensity in our prepared medium; nevertheless, ***E. coli*** (Gram- bacterium) showed a weak or limited growth in this medium figure(7), this limited growth of ***E. coli*** in the solidified enriched medium may interpreting as a result of absence in one or more essential substances for its growth in this medium.

The progressive growth of ***P. aeruginosa*, *P. vulgaris*** and ***K. pneumoniae*** in the enriched medium figure (5 and 6) interpreting as that the components of this experimental medium supplies these bacteria with its growth requirements, so it could be consider this medium very suitable for their growth and maintenance also since the growth of these types continue for at least (4-5) days in this medium, in addition, it could considered a good substitute for the nutrient agar which used for the growth of these types.

The chemical components of our enriched solidified medium is free of the animal protein, blood, serum and any other enriched components from animal origin which found in the blood agar (the suitable medium for ***S. aureus*** and ***S. pneumoniae***), so that, there is a slight possibility of expedience of the prepared medium to the growth of these bacteria. In spite of that, there was a moderate growth of ***S. aureus*** in the enriched solidified medium, may be because this type of bacteria has a high resistance against unsuitable conditions; while the genus ***S. pneumoniae*** showed a limited growth, because this type needs a special medium meet their nutritional and environmental requirements and provide it with a suitable growth components that similar to those in their infected foci which is absent in this experimental medium.

The color of the enriched solid medium before the cultivation with any bacteria is brown (figure 4); it is not changed after the growth of each one of the six types. The color of the colonies of the certain type was different each from the other in the enriched medium, the colonies takes a color ranges between grayish (in ***P. vulgaris*** and ***K. pneumoniae***) or brown to grayish (in ***P. aeruginosa***) to transparent (in ***S. aureus*, *E. coli*** and ***S. pneumoniae***), (Table-2).

The biochemical tests confirmed that the diagnosed samples are the same types of the selected bacteria, this step improve that the obtained growth was not because of any contamination in the medium or during the cultivation of the bacteria.

As a conclusion, it could use the liquid form of this extract as a liquid culture medium directly instead of the nutrient broth and get acceptable results. If the extract used as an enriched substance for the agar-agar it produces a suitable source for nutrients and promote the growth of the certain type of bacteria. It could not consider this medium as a selective medium because it provides a rich nutritional environment for many types of microbes since it supports the growth of many types of (Gram+) and (Gram-) bacteria. It also could not consider as a differential medium because it isn't providing colonies with different color according to the type of the cultivated bacteria. Generally, the medium represents a cheep, simple prepared natural medium used for the

cultivation of different type of bacteria and it could substitute this medium instead of the routine media in the cultivation of many types of bacteria such the types used in this research.

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