Antibacterial activity of ethanol and aqueous extracts of some Perennial plants against three gram negative pathogenic bacteria from Koya city – Kurdistan Region - Northern Iraq

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Abstract

The aim of this investigation was to determine whether both ethanol and aqueous extracts of different parts of three Perennial plants (*Dodonaea viscosa*, *Eucalyptus* sp. and *Pinus* sp.) could provide the biological activity to inhibit the growth of three gram negative pathogenic bacteria include: *Eschericia coli*, *Pseudomonas aeruginosa* and *Salmonella typhi.* 20 grams of the plant parts powder were extracted with 200 ml of ethanol and 40 grams were extracted with 160 ml of distilled water. MIC was estimated for ethanol and aqueous. Antibacterial activity assay of the ethanol and aqueous extracts was carried out disk diffusion method. Pure colonies of test bacteria were transferred to nutrient broth and incubated overnight at 37C˚ and turbidity of prepared inoculums were adjusted equal to that 10^6 CFU/ml (standardized by 0.5 McFarland standards) and 100µl of inoculum was spread on Muller-Hinton agar medium by using a sterile glass spreader. For control, discs were impregnated with sterile water or absolute alcohol (control negative) and also standard antibiotic discs (ciprofloxacin 10µg/disc, Ampicillin 10µg/disc and cloxacillin 10µg/disc, Tobramycin 10µg/disc) as a control. Interestingly in our study we have observed promising antimicrobial activity against studied

bacteria. It is worth mentioning that most of the results of aqueous and ethanolic extracts were distinct because they cannot appearing in some large inhibition zone, but it was interesting because it was close to the positive control , where inhibition percentage arrived to more than 90%. Suggest both extracts of *D.viscosa* shoots against *P. aeruginosa*, *Eucalyptus* sp. leaves against *E.coli* and *S. typhi* , ethanolic extract of pine cone against *P. aeruginosa* and aqueous extract of pine leaves against *E.coli* , as a good antibacterial" .

Keywords: Antibacterial, gram negative bacteria, *Dodonea Viscosa* ,*Eucalyptus* sp.,*Pinus* sp.

الفعالية المضادة للبكتريا للمستخلصات الكحولية والمائية لبعض النباتات المعمرة تجاه ثلاثة أنواع من البكتريا المرضية السالبة لصبغة كرام والمعزولة من مدينة كويا- إقليم كردستان- شمال العراق

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الخلاصة

ان الهدف من هذا البحث هو تحديد ما إذا كان كل من المستخلص الكحولي والمائي من أجزاء مختلفة من ثلاثة نباتات معمرة وهي (الديدونيا، اليوكالبتوس والصنوبر) يمكن أن تكون ذات فعالية بايلوجية لتثبيط نمو ثلاثة انواع من البكتيريا المرضية السالبة لصبغة كرام وهي *Eschericia coli, Pseudomonas aeruginosa, Salmonella typhi*. وزن 20 غراما من مسحوق أجزاء النباتات اضيف الى 200 مل من الايثانول و 40 غراما مع 160 مل من الماء المقطر. وتم تقدير التركيز المثبط الادنى للمستخلص الكحولي والمائي لها. وأجري تقييم الفعالية المضادة للبكتيريا للمستخلصات الكحولية والمائية بطريقة الأقراص. تم نقل المستعمرات النقية من البكتيريا الى وسط المرق المغذي وحضنت لمدة 24 ساعة في 37م° وتم ضبط عكورة اللقاح بما يساوي 10 ^{6} خلية / مل (باستخدام محلول ماكفرلاند بتركيز 0.5) وتم نشر 100مايكروليتر من اللقاح على وسط مولر هينتون باستخدام ناشر زجاجي معقم. وغمست أقراص ورقية بالماء المعقم أو الكحول المطلق (كعينات سيطرة سالبة) واستخدمت أقراص المضادات الحيوية القياسية وهي (سيبروفلوكساسين ، أمبيسلين ، كلوكساسيلين، توبرامايسين) بتركيز 10 مايكروغرام/ قرص (كعينات سيطرة).من المثير للاهتمام في دراستنا هذه ملاحظة النشاط المضاد للميكروبات بصورة واضحة ضد البكتيريا التي شملتها الدراسة. ومن الجدير بالذكر أن معظم

تراكيز المستخلصات المائية والكحولية كانت ذات فعالية واضحة، حتى و أن لم تظهر في بعضها مناطق تثبيط كبيرة، لكنه كان كان قريبا من السيطرة الإيجابية، حيث وصلت نسبة تثبيط بعضها الى أكثر من 90٪. أظهرت المستخلصات المائية والكحولية لنبات الديدونيا فعالية واضحة تجاه بكتريا الزوائف الزنجارية، وأظهر مستخلص اوراق اليوكالبتوس فعالية تجاه بكتريا الايشريشيا القولونية والسالمونيلا التيفوئية، واظهر المستخلص الكحولي لمخروط الصنوبر فعالية ضد الزوائف الزنجارية واظهر المستخلص المائي لأوراق الصنوبر فعالية ضد الايشريشيا القولونية.

الكلمات المفتاحية: مضادات البكتريا، البكتريا السالبة لصبغة كرام، الديدونيا، اليوكالبتوس، الصنوبر

Introduction

Plants have attracted researchers all over the world as a source of medicinal treatment because of the active compounds that present in their parts. Recently these plants of medicinally importance are increasingly being investigated by researchers because of their antimicrobial activity. The antimicrobial activities attributed to compounds synthesized by plants which are known by their active ingredients for instance the phenolic compounds which are part of the essential oils "**(1)**. Plants are rich in a wide variety of secondary metabolites such as tannins, alkaloids and flavonoids, which have been found *in vitro* to have antimicrobial properties **(2)**. In addition 'ethno-directed sampling' of species used in traditional medicine has proven far more fruitful in the identification of new drugs compared to random screening **(3)** Several studies have been conducted on antimicrobial activity of plants in different parts of the world in an effort to discover new antimicrobial compounds from various plants and their species. These novel compounds may represent an alternative to synthetic chemicals such as drugs and antibiotics, which may exhibit side effects. The development of bacterial resistance to presently available antibiotics has necessitated the need to search for new antibacterial agents. The widespread and indiscriminate prescription of antibiotics has resulted in the emergence of a number of drug-resistant bacteria **(4)**. *Eschericia coli* strains are examples of multi resistant bacteria that are becoming an alarming problem within the healthcare system **(5** and **6)**. There is a strong necessity for the development of new drugs for the cure of infections provoked by these resistant and multi-resistant bacterial species **(7)**. These bacteria are associated with a number of infections including: UTIs, lower and upper respiratory tract infections and typhoid

fever. Moreover, these pathogenic bacteria are capable of elaborating several virulent factors including the formation of biofilms on colonized surfaces **(8** and **9)**. The aim of this investigation was to determine whether both ethanol and aqueous extracts of different parts of three Perennial plants (*Dodonaea viscosa* , *Eucalyptus sp*. and *Pinus sp*.) could provide the biological activity to inhibit the growth of three gram negative pathogenic bacteria *Eschericia coli , Pseudomonas aeruginosa* and *Salmonella typhi* .

Materials & Methods

1- Plant materials

Scientific Classification of" Selected plants.

1- *Eucalyptus* sp. **(10)**	2- *Dodonaea viscosa* **(11)**	3- *Pinus* sp. **(12)**
Kingdom: Plantae Order: Myrtales Family: Myrtaceae Genus: *Eucalyptus* Species : sp. *Binomial Name:* *Eucalyptus* sp.	Kingdom: Plantae Order: Sapindales Family: Sapindaceae Genus: *Dodonaea* Species: *viscosa* *Binomial Name:* *D.viscosa*	Kingdom: Plantae Order: Pinales Family: Pinaceae Genus: *Pinus* Species: sp. Binomial Name: *Pinus* sp.

Preparation of plant material.

The fresh leaves, inflorescence and shoots of (*Dodonaea viscosa* and *Eucalyptus* sp.) , the fresh leaves, cones and shoots of (*Pinus* sp.) were harvested , rinsed with tap water and air dried under shade and reduced to coarse powder and then micronized to fine powder using the electric blender .The powder was stored in an airtight paper bag until required .

Preparation of the ethanolic extracts.

The preparation of the different parts extracts were performed following the methods described by **(13)**. 20 grams of the powder were extracted with 200 ml of solvent (ethanol) contained in a 500 ml sterile conical flask and covered with cotton wool plug and wrapped

with aluminum foil. Extraction was allowed to proceed for 24 h in cooler. The extract was filtered using a clean muslin cloth and then whatman No. 1 filter paper. The filtrate was then evaporated to dryness using a rotary evaporation attached to a vacuum pump.

Preparation of the aqueous extracts.

The preparation of the different parts extracts were performed following the methods described by **(14)**. 40 grams of the powder were extracted with 160 ml of solvent (distilled water) contained in a 500 ml sterile conical flask and covered with cotton wool plug and wrapped with aluminum foil. Extraction was allowed to proceed for 24 h in cooler. The extract was filtered using a clean muslin cloth and then Whatman No. 1 filter paper".

2- Pathogenic Bacteria.

Bacterial isolates:

Eschericia coli (ATCC: 25218)

Salmonella typhi (ATCC: 14028)

Pseudomonas aeruginosa (ATCC: 27853).

Those isolated bacteria obtained from laboratory of general microbiology and a laboratory of medical bacteriology / department of Medical Microbiology / Faculty of Science and Health / Koya university.

Antibacterial Assay

Antibacterial activity assay of the ethanol and aqueous extracts was carried out disk diffusion method against test bacteria according to **(15)**. Pure colonies of test bacteria were transferred to nutrient broth and incubated overnight at 37C˚ and turbidity of prepared inoculums were adjusted equal to that 10^6 CFU/ml (standardized by 0.5 McFarland standard) and 100µl of inoculum was spread on Muller-Hinton agar medium by using a sterile glass spreader. Sterile filters paper (Watchman No. 1, diameter 5 mm) were impregnated in 40 µl from both extracts and placed on the culture medium (MHA). For control, discs were impregnated with sterile water or absolute alcohol (control negative) and also standard antibiotic discs (ciprofloxacin 10µg/disc, Ampicillin & cloxacillin 10µg/disc, Tobramycin 10µg/disc) .The prepared disks were placed on lawn cultures of the bacteria. The plates were left at room temperature for one

hour to allow the diffusion of extract into the medium, and then were incubated at 37˚C for (24-36) hours to allow maximum growth of the microorganisms. The inhibition zone diameter around each disk was measured (mm). The assay was repeated twice and mean of the experiments was recorded.

Determination of MIC value of ethanol and aqueous extract.

"Minimum Inhibitory Concentration (MIC) of the ethanol and aqueous extract against the tested bacteria was determined using serial two fold dilutions of ethanol plants extract with 100 µl. of fresh cultures (1.5 * 10^5 CFU/ml standardized by 0.5 McFarland standard) in each well from Micro titer plate (BRAND plates®, Germany) . The concentration of the ethanol extracts were ranged from 62.5 mg/ml to 1000 mg/ml, but the concentration of the aqueous extracts were ranged from 78.13 mg/ml to 1250 mg/ml. Each assay was run in triplicates. The inoculated plates were incubated for 37 °C for 24 hours. After incubation period, the MIC values were determined by observed the turbidity of the wells in the micro titer plate. Well of the micro titer plate that showed no turbidity was interpreted as no growth of the tested bacteria. The MIC was defined as the lowest concentration of plant extracts that can inhibit the growth of the tested bacterial".

Statistical analysis

All determinations were carried out in twice replicate and the values are mean ± standard error (S.E).

Results and Discussion

Getie *et al*., (**16**) reported the absence of *Dodonaea viscosa* activity against gram negative organisms, but interestingly in our study **(Table 1)** we have observed promising antimicrobial activity against studied bacteria .Antibacterial activity of ethanolic and aqueous extracts of different parts of *D.viscosa* are presented in table 1 which showed that there some differences efficiency among different data of ethanolic extract were the height data showed with ethanolic extract of shoots against *P.aeruginosa* which arrived to 14.5 mm , were there are some observations with aqueous extract showed attractive results when gives inhibition zone arrived to 13 mm compared with positive control 11 mm by shoots extract against

P.aeruginosa. Khurram *et al.*, **(17)** reported the promising activity against gram negative bacteria. The crude ethanolic extract and aqueous fractions of *D.viscosa* were analyzed for antibacterial potential against three gram negative bacteria: *E.coli*, *S.typhi*, and *P.aeruginosa*. Preliminary screening showed inhibition against *E.coli* and *P.aeruginosa* **(18**and **19)**.

Table 1. Antibacterial activity of plant parts extract of *Dodonaea viscosa* against pathogenic bacteria.

Plant Parts	Pathogenic Bacteria	Ethanolic Extract (inhibition zone/mm)	MIC mg/ml	Aqueous Extract (inhibition zone/mm)	MIC mg/ml
Leaves	***S. typhi***	13.5 ± 0.51	62.5-1000	9 ± 1	78.13-1250
	E.coli	11 ± 1	62.5-1000	11.5 ± 1.5	78.13-1250
	P.aeruginosa	14.5 ± 0.51	62.5-1000	12± 2	78.13-1250
inflorescence	***S. typhi***	12 ± 0	62.5-1000	N.I.	78.13-1250
	E.coli	11 ± 1	62.5-1000	7 ± 1	78.13-1250
	P.aeruginosa	10 ± 0	62.5-1000	12 ± 0	78.13-1250
Shoots	***S. typhi***	13 ± 1	62.5-1000	7.5 ± 0.51	78.13-1250
	E.coli	10 ± 0	62.5-1000	8.5± 1	78.13-1250
	P.aeruginosa	8 ± 0	62.5-1000	13 ± 1	78.13-1250

Data given are mean of two replicates ± S.E., N.I= No Inhibition, MIC= Minimum Inhibitory Concentration

The results in **table2** showed the biological activity of *Eucalyptus* sp., there are more attractive results showed, where the observation data appeared height inhibition of ethanolic and aqueous extract of leaves against *E.coli*, which arrived, to 17.5 and 16 mm respectively compared with standard antibiotic that arrived to 17 and 10 mm respectively. "The ability of the crude extracts to inhibit the growth of recalcitrant bacteria as those used in this study is in agreement with previous reports of the antibacterial activities of other *Eucalyptus* species **(9** and **20)**. Natural products, such as a plant extract, either as pure compounds or as standardized extracts, provide unlimited opportunities for control of microbial growth owing to their chemical diversity. Besides antimicrobial, several plants are being used in different areas of

human health such as traditional medicine, functional foods, dietary supplements and recombinant protein manufacturing. Phytochemicals, especially flavonoids, polyphenols, anthocyanin and carotenoids, share the major market **(21)"**.

Table 2. Antibacterial activity of plant parts extract of *Eucalyptus* sp. against pathogenic bacteria.

Plant Parts	Pathogenic Bacteria	Ethanolic Extract (inhibition zone/mm)	MIC mg/ml	Aqueous Extract (inhibition zone/mm)	MIC mg/ml
Leaves	***S. typhi***	15 ± 3	62.5-1000	14 ± 1	78.13-1250
	E.coli	17.5 ± 0.51	62.5-1000	16 ± 1	78.13-1250
	P.aeruginosa	12 ± 2	62.5-1000	15.5 ± 0.51	78.13-1250
inflorescence	***S. typhi***	14 ± 1	62.5-1000	13.5 ± 0.51	78.13-1250
	E.coli	16.5 ± 1.5	62.5-1000	14.5 ± 0.51	78.13-1250
	P.aeruginosa	15.5 ± 0.51	62.5-1000	13 ± 1	78.13-1250
Shoots	***S. typhi***	14 ± 2	62.5-1000	11 ± 1	78.13-1250
	E.coli	16 ± 2	62.5-1000	11.5 ± 0.51	78.13-1250
	P.aeruginosa	12 ± 0	62.5-1000	12 ± 1	78.13-1250

Data given are mean of two replicates ± S.E., MIC= Minimum Inhibitory Concentration

Antibacterial activity of ethanolic and aqueous extracts of different parts of *Pinus* sp. are presented in **Table 3**. Highly significant antibacterial activity was observed in ethanolic extract of cones against *P.aeruginosa* compared with other results, but the result which observed in aqueous extract of leaves showed highest inhibition zone against *E.coli* compared with positive control and others **(22)**. These results show that the aqueous extract of pine leaves are the best among the other extracts and the aqueous extract better than ethanolic and this is the most security when compared the water with ethyl alcohol.

Table 3. Antibacterial activity of plant parts extract of *Pinus* sp. against pathogenic bacteria.

Plant Parts	Pathogenic Bacteria	Ethanolic Extract (inhibition zone/mm)	MIC mg/ml	Aqueous Extract (inhibition zone/mm)	MIC mg/ml
Leaves	***S. typhi***	N.I.	62.5-1000	11 ± 1	78.13-1250
	E.coli	10 ± 1	62.5-1000	11 ± 1	78.13-1250
	P.aeruginosa	11 ± 1	62.5-1000	9.5 ± 1	78.13-1250
Cones	***S. typhi***	N.I.	62.5-1000	9 ± 1	78.13-1250
	E.coli	12 ± 2	62.5-1000	9 ± 1	78.13-1250
	P.aeruginosa	13 ± 1	62.5-1000	10 ± 0	78.13-1250
Shoots	***S. typhi***	N.I.	62.5-1000	10 ± 1	78.13-1250
	E.coli	10 ± 1	62.5-1000	9.5 ± 1.5	78.13-1250
	P.aeruginosa	10 ± 0	62.5-1000	10.5 ± 0.51	78.13-1250

Data given are mean of two replicates ± S.E., N.I= No Inhibition, MIC= Minimum Inhibitory Concentration.

In order to more discuss the findings and to clarify the percentage inhibition zone, the figure 1-3 shows that there are clear differences between the alcoholic and aqueous extract against studied bacteria. The maximum percentage inhibition against *S.typhi* was recorded by leaves and inflorescence extract of *Eucalyptus* sp. **(figure1)** as compared with other extracts and positive control which arrived to (78.95 & 73.68) % for ethanolic extract respectively and (73.68 & 71.00) % for aqueous extract respectively , at the same time there are other activity of ethanolic extract of *Eucalyptus* shoots and *Dodonaea* leaves (73.68 & 71.00) % respectively **(23)**.The ethanolic extract of *D. viscosa* leaf has anti-bacterial effect against gram negative bacteria **(24)** , therefore to follow the inhibition percentage of biological effectiveness of the studied plants against *E. coli* the **figure2** shows that the highest percentages of inhibition against it was at ethanolic extract of *Eucalyptus* leaves (92.11 %) , but there are attractive results compared with positive control , where the ethanolic extract of inflorescence and shoots of same plant appeared good inhibition percentage (86.84 & 84.21) % respectively , so the highest inhibition percentage of aqueous extract arrived to (84.21&76.32) % respectively for leaves and inflorescence extract of *Eucalyptus* , the above

results is considered as attractive results compared with positive control which arrived to 71.05% at its best inhibitory cases. According to **(16** and **25)** the crude extract of *Dodonea viscosa* has no activity against *E. coli.* . Studied plants were appeared different activity against *P.aeruginosa* as **figure3** showed, "where the highest zone of inhibition showed by leaves aqueous extract and inflorescence ethanolic extract of *Eucalyptus* (81.58 %) compared with other data included positive control. It is worth mentioning that most of the results of aqueous and ethanolic extracts were distinct because they are, even if it was cannot appearing in some large inhibition zone, but it was interesting because it was close to the positive control. May be attributed the observed antimicrobial activities to the presence of some bioactive compounds like alkaloids tannins, saponins, terpenes, essential oils and amongst others, several authors have linked the presence of these bioactive compounds to the antimicrobial properties of crude plant extracts **(9 , 26 , 27, 28** and **29)**. Effectiveness of plants as antimicrobial agents is hinged on their mode of action in the body , generally, plant products have been demonstrated to have tropism for specific organs or systems in the body with resultant multiple effects on the body **(9** and **30)"**.

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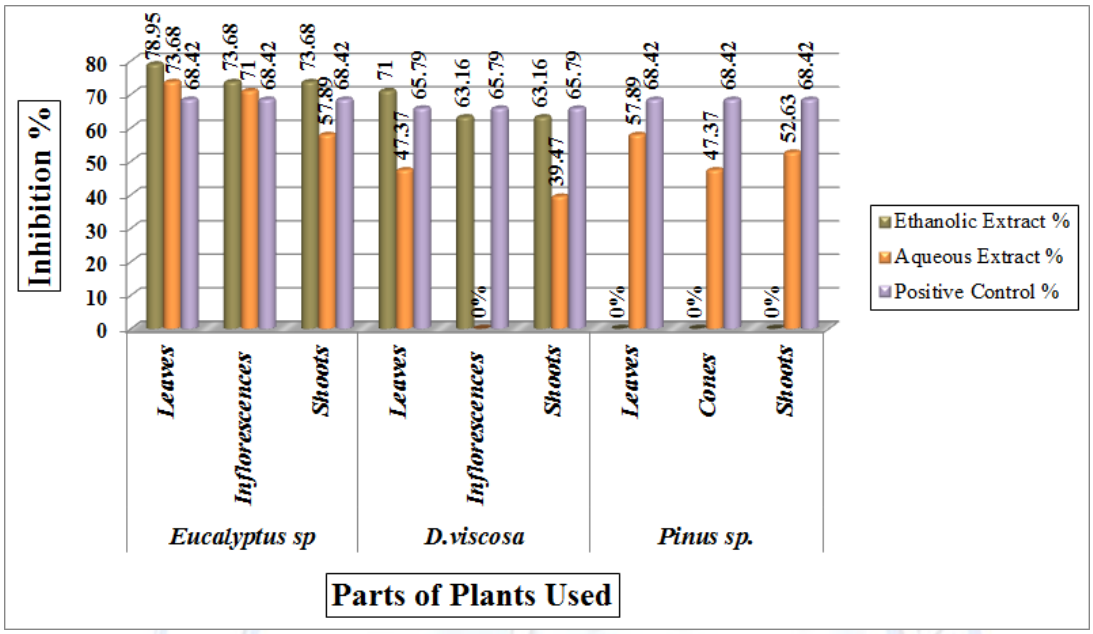

Figure 1. Inhibition percentage of three different parts extract from plant used against *Salmonella typhi*.

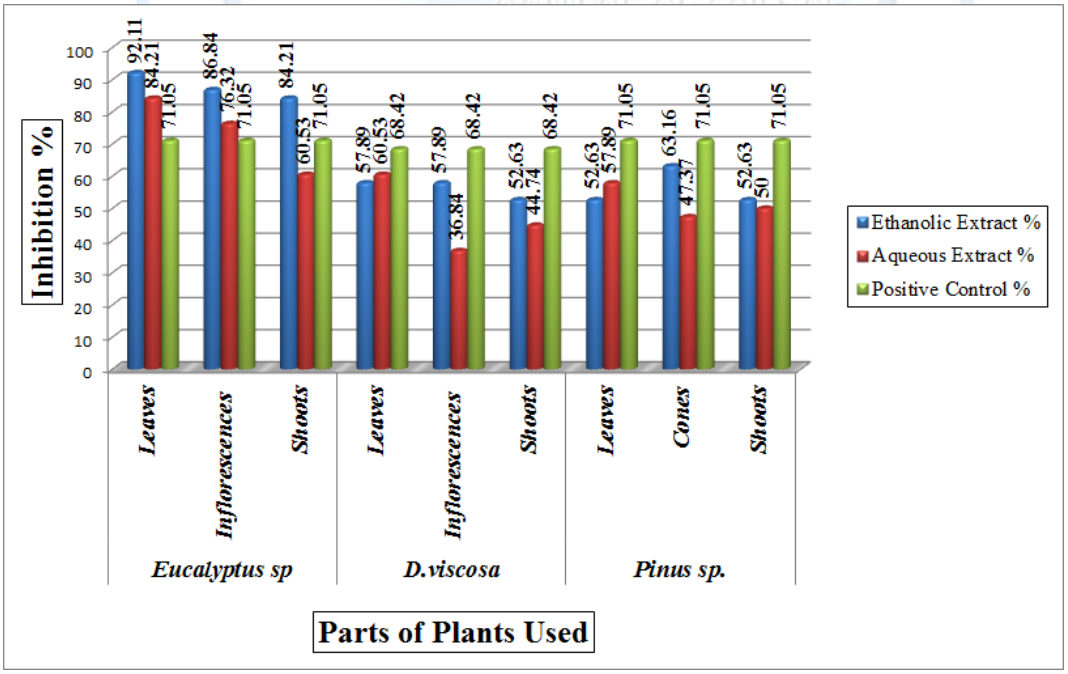

Figure 2. Inhibition percentage of three different parts extract from plant used against *Escherichia coli.*

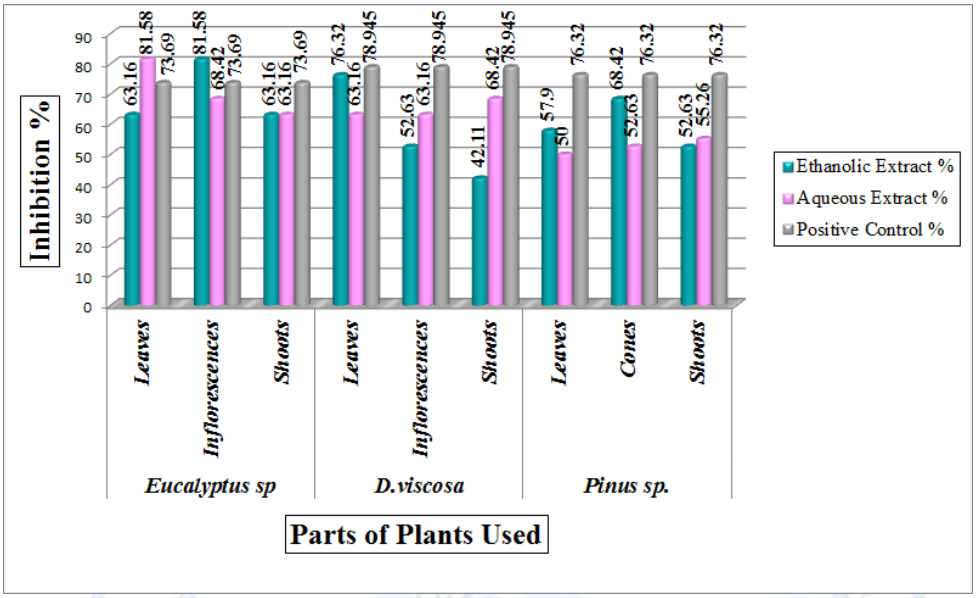

Figure 3. Inhibition percentage of three different parts extract from plant used against *Pseudomonas aeruginosa*.

Conclusions

Suggest extracts of *D.viscosa* shoots were active against *P. aeruginosa*, *Eucalyptus sp*. leaves were active against *E.coli* and *S. typhi*, while ethanolic extract of pine cone were active against *P. aeruginosa*. also aqueous extract of pine leaves against *E.coli*. As a good antibacterial and the fact that the studied plants possesses many medicinal factor makes it a very useful plants, and the extracts could be useful in therapeutic treatment, but this has to be substantiated by *in vivo* experiment.

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