International Journal of Biology Sciences

ISSN Print: 2664-9926
ISSN Online: 2664-9934
Impact Factor: RJIF 5.45
IJBS 2023; 5(1): 87-91
www.biologyjournal.net
Received: 14-10-2022
Accepted: 18-11-2022

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Effect of *Rosmarinus officinalis* L. extracts on bacteria isolated from conjunctivitis in Kirkuk City, Iraq

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DOI: https://dx.doi.org/10.33545/26649926.2023.v5.i1b.150

Abstract
Conjunctivitis is an eye disease that causes swelling or Conjunctival inflammation. It is one of the most common ocular diseases worldwide. This work aimed to study the effect of *Rosmarinus officinalis* (alcoholic and aqueous) extracts against isolated bacteria from conjunctivitis persons, further evaluation of the phytochemical test, antioxidant activity test, and sensitivity test for bacterial isolates. 50 samples were collected from conjunctivitis patients were visited private medical laboratories in Kirkuk city from 15 July to 1 December 2022 by using sterile transport swabs, planted into several culture media incubated under aerobic and anaerobic conditions at 37 °C for 24-48 hr. diagnosed with biochemical tests and confirmed with the API system. Alcoholic and aqueous rosemary extracts were prepared with three concentrations 10%, 20%, and 30%. Three tests were conducted: sensitivity test, a phytochemical test, and an antioxidant activity test. Out of 50 samples, 31 (62%) were positive culture. While 19 cases gave a negative result to the culture. *Staphylococcus aureus* was the most prevalent (11 isolates). Then *Streptococcus viridance* (8 isolates), *Pseudomonas aeroginosa* was prevalent (7 isolates), (3 isolates) of *E. coli* and *Klebsiella pneumonia* was (2 isolates). Regarding the extracts' inhibitory effect, the alcoholic extract was effective in strains than aqueous extract, and the inhibitory activity increased with increasing concentrations. Gram-positive bacteria were more susceptible to extracts of rosemary than Gram-negative bacteria. *Staphylococcus aureus* was shown to be the most sensitive to rosemary extracts, while *Pseudomonas aeroginosa* was found to be the least sensitive. The phytochemical reaction in alcohol extract revealed 8 phytochemical components, whereas revealed 5 phytochemical components in aqueous extract. In addition, alcohol extract had higher antioxidant activity than aqueous extract.

Keywords: Conjunctivitis, Rosmarinus, *Staphylococcus aureus*, antioxidant activity, alcohol extract

Introduction
Conjunctivitis is the most prevalent ocular disease worldwide [1]. It is a conjunctival mucosa inflammation with significant economic and social consequences [2]. It is classified as chronic, acute or severe acute [3]. It may cause a number of signs and symptoms, such as pus production, lid oedema, hyperemia of the conjunctiva and possibly impaired vision. The Pathogenic bacteria can enter the body via the environment or blood infection [4, 5]. Bacteria cause 50-70% of contagious conjunctivitis [6]. Infectious conjunctivitis is most common in elders and kids but can also affect adults and newborns [7]. The majority of them have a self-limiting course of conjunctival inflammation with mucosal discharge. However, in some cases, the presence of pus may speed up the deterioration of the eye [7, 8]. There are multiple reports of bacterial profiles and antibiotic sensitivity in cases of eye infection with variable outcomes [9, 10]. Although most cases of conjunctivitis are self-limiting, antibiotic treatment has been demonstrated to minimize the severity of the symptoms and discomfort and the transmission of infection [11-14]. The prevalence of bacterial conjunctivitis, common infections, and antibiotics sensitivity varies among countries [8, 15-18]. Also, there is a shifting trend in the bacteria's sensitivity and resistance towards antibiotics available in the past decade [8]. The indiscriminate use of antibiotics has increased antibiotic resistance. Besides, sometimes, these antibiotics cause allergies and immunosuppression. Therefore, plant extracts are safer for human health and the environment [19-21]. So it was going to plants, for example, Rosemary, Rosemary (*Rosmarinus officinalis* L.).

For a long time, as a spice and medical herb, which had known for a long time, it's a member of the *Lamiaceae* family and receives more attention due to its anti-inflammatory, antibacterial and antioxidant components [22]. It is originally grown in the south of Europe. Because of its attractive flavor, it is employed as a flavor and spice agent in food preparation and recently as an antibacterial agent [23-25]. The purpose of the research was to study the effect of *Rosmarinus officinalis* (aqueous and alcoholic) extracts against bacteria isolated from conjunctivitis patients, Also evaluation of the phytochemical test, antioxidant activity test, and sensitivity test for bacterial isolates.

Materials and Methods

The study was conducted at private medical laboratories in Kirkuk city from 15 July to 1 December 2022.

Bacterial Isolation and Identification: 50 samples were collected from conjunctivitis patients by using sterile transport swabs. Samples cultured on blood agar, chocolate agar, and McConkey, s agar, incubated under aerobic and anaerobic conditions at 37° C for 24-48 h. Organisms were identified using microscopic and biochemical tests [26, 27], all isolates were confirmed using the API system (Bio-Merieux, France).

Rosemary extract: The rosemary herb was obtained from local markets, washed with clean sterile distilled water then allowed to air dry and grind smoothly.

Aqueous extraction: 100 gm. of the herb powder was mixed with 1 l of distilled water. The extract was filtered with filter paper and gauze after 1 hr. of stirring and then it was filtered by centrifuge according to the [28] method.

Alcoholic extraction: 1 l of 80% methanol was mixed with 100 gm. of the herb powder. The subsequent steps for the aqueous extraction were the same after 22 to 24 hr. of stirring [28].

Preparation of concentrate solutions: Different concentrations were prepared for each extract (10%, 20% and 30%) [29].

Antibacterial test: The disc diffusion method was utilized to determine the antibacterial activity of rosemary extract. Briefly, bacterial suspensions were spread onto Mueller Hinton Agar plates using a clean, sterile cotton swab. Sterile paper (6mm in diameter) discs were impregnated with 10µl of each concentrate and placed on the inoculated dishes. Control disks were also manufactured. Plates were maintained at 4°C for 2 hr. before incubating at 37°C for 24 hr. The millimeter scale was used to measure the inhibitory zones. Each test was carried out in triplicate [30].

Phytochemicals screening test: A phytochemical analysis test is utilized to identify the phytochemicals, including saponin, terpenoids, tannins, Phlobatanins, tannins, Cardiac glycosides, alkaloids, phenols, and flavonoids. This test was performed for each extract separately, According to the standard operating procedures described by Soundararajan and Coworkers, 2017, and Kumar and Coworkers 2018 [31, 32], in order to check for the presence of phytochemicals.

Tannins: A small amount of each extract was combined with a few drops of a 10% neutral lead acetate solution. The formation of a white to pale yellow precipitate indicates the existence of tannins.

Saponin: A little amount of each extract was mixed with two to three drops of distilled water and shook well. The formation of semi-permanent foam indicates the existence of saponins.

Flavonoids: A little amount of each extract was mixed with a few drops of the FeCL3 solution. The presence of flavonoids is indicated by the creation of a green to black color.

Terpenoids: A little amount of each extract was mixed with 2drops of chloroform, and 1 ml of concentrated hydrochloric acid, after 2 mn of heating, the color changed to reddish brown indicating the presence of terpenoids.

Alkaloids: 1 ml of Mayer's reagent was combined with a small amount of each extract in a test tube. The formation of yellow cream precipitates indicates the presence of alkaloids.

Phlobatanins: A 2% hydrochloric acid solution was heated up with 10 mL of crude extracts. The presence of phlorotanin was demonstrated by the red precipitate [33].

Cardiac glycosides: 10 ml of crude extract was shaken with 1 ml of glacial acetic acid. 1 drop of concentrated sulfuric acid and 1 drop of ferric chloride were added. Cardiac glycosides were seen in the top layer as a blue-green colour and as a reddish-brown colour at the confluence of the two layers [33].

Ferric chloride test for phenol: 5 ml of the crude extracts were mixed with 15 ml of distilled water. Next, Several drops of 5% neutral FeCl3 solution were added. The formation of a dark green colour indicates the presence of phenolic substances [33, 34].

Antioxidant activity test: This assay combines the antioxidant activity of Rosmarinus *officinalis* extracts with the scavenging activity of stable 2, 2-diphenyl-1 picrylhydrazyl (DPPH) free radicals [35]. This test was conducted independently for each extract, three volumes (50-100-150µl) of each extract were put into the test tubes, and 1 ml of distilled water was used to finish the volume, then 1 ml of DPPH solution was added to each test tube, mixed and incubated for 30 minutes at room temperature. Ascorbic acid (0.03%W/V) was utilized as the control. A spectrophotometer was used to measure the absorbance (A) at 517 nm. The following equation was used to calculate the DPPH free radicals inhibition in percent (1%) [36] 1%= (Ac – As) / Ac * 100.

Ac: The control's absorbance.

As: The sample's absorbance.

Statistical Analysis: Mean, number, percentage and part per million.

Results and Discussion

A total of 50 samples from the conjunctivitis infections were cultured during the study period. 31 (62%) were positive culture. While 19 cases gave a negative result to the culture, viruses, fungus or others may cause. 19 (61.29%) of bacteria were gram-positive, while 12 (38.71%) isolates were negative, as shown in Table 1.

Table 1: Bacterial isolates from conjunctivitis patients

Positive samples	%	Genus	Number	%
31from 50 samples	62	Gram-positive organisms	19	61.29
		Staphylococcus aureus	11	35.48
		Streptococcus viridance	8	25.80
		Gram-negative organisms	12	38.71
		Pseudomonas aeroginosa	7	22.58
		Klebsiella pneumonia	2	6.45
		E. Coli	3	9.67
		Total	31	100

Staphylococcus aureus was the most prevalent (11 isolates). Then *Streptococcus viridans* (8 isolates), *Pseudomonas aeruginosa* was prevalent (7 isolates), (3 isolates) of *E. coli* and *Klebsiella pneumonia* (2 isolates). These results agree with [37-39], who stated that Gram-positive bacteria were more percentage than negative bacteria. Gram-negative bacteria are less prevalent than Gram-positive bacteria, but they are more diverse as compared to Gram positives.

Antibacterial test: The rosemary extracts showed that an alcoholic extract affected more than the aqueous extract, as shown in Table (2), and different concentrations of the herbal extract determined the inhibitory effect of rosemary extract. Also, the outcomes demonstrated that the inhibitory effect depends on the dose by raising the extract's concentration, revealed a reduction in growth; the highest inhibition was recorded at concentration 30%. The effectiveness of alcoholic rosemary extract may contain compounds: tannins, flavonoids, and saponins. These compounds have inhibitory efficiency for a group of microorganisms, including bacteria and fungi [40]. Besides that, Rosemary extract was more effective against Gram-positive bacteria than Gram-negative bacteria, and these results are in agreement with Grosvenor and Gray [41]. They discovered that gram-positive bacteria were the most susceptible when compared with gram-negative bacteria. Higher activity of the extract and its components against bacterial species were reported by Rico and Rios [42]. They found that antimicrobial activity differences might be due to the difference in bacterial cell wall constituents. *Staphylococcus aureus* was discovered to be the most susceptible bacteria to rosemary extracts, which was agreed with [43], whereas the least susceptible was *pseudomonas aeroginosa*, as shown in table (2).

Table 2: The mean diameter of the inhibition zones (mm) of different concentration of aqueous extract and alcoholic extract on bacterial isolates

Bacterial species	The mean diameter of the inhibition zones (mm)						
	Aqueous extract of rosemary			Alcoholic extract of rosemary			Gentamicin as control
	10%	20%	30%	10%	20%	30%	
Staphylococcus aureus	6.9	8.2	9.7	7.6	8.5	10.2	22
Streptococcus viridance	5.4	6.8	7.4	5.9	7.1	7.7	23
Pseudomonas aeroginosa	4.3	5.2	6.3	4.9	5.3	6.8	17
Klebsiella pneumonia	5.6	5.8	6.4	5.9	6.7	7.2	15
E. Coli	5.4	5.8	6.3	6.5	7.4	7.8	12

Phytochemical screening test: Both the aqueous and the alcoholic extracts performed separate screening tests for phytochemicals. The outcomes are shown in (Table 3). All of the chemical components' reactions in the alcoholic extract were positive (Saponins, Tannins, Phlobatanins, Flavonoids, Cardiac glycosides, Terpenoids, Alkaloids and Phenols). Whereas detection of Tannins, Saponins and Flavonoids showed a negative result in the aqueous extract but the other tests showed positive results.

The low antibacterial effect of the aqueous extract could result from the fewer phytochemicals (flavonoids, saponins, and tannins) derived with aqueous extraction in comparison to alcohol extraction. In the present research, alcoholic extract included more phytochemicals than aqueous extract. This explains why the alcoholic extract had the biggest zone of inhibition in the antibacterial tests because ethanol is the greatest solvent for the active chemicals isolated from plant as compared to distilled water, which was utilized in the aqueous extract as a solvent. Similarly, Edrah and Coworkers 2017 [44] extracted (saponins, tannins, flavonoids and alkaloids); these constituents are responsible for the antibacterial activities against microbes. This result is in line with extracts of *Rosmarinus officinalis*, which included active substances with effective antibacterial effects.

Table 3: Phytochemical screening results

Chemical component	Aqueous extract	Alcoholic extract
Tanins	-	+
Saponins	-	+
Phlobatanins	+	+
Flavonoids	-	+
Trepenoids	+	+
Cardiac glycosides	+	+
Alkaloids	+	+
Phenols	+	+

+: present; -: absent.

According to the study's findings, the existence of Chemical components in the extracts could have biological effects on

five types of bacteria isolated from conjunctivitis patients. On both Gram-positive and Gram-negative bacteria, the aqueous and alcohol extracts of *Rosmarinus officinalis* had an inhibitory impact. The antibacterial activity of the alcoholic extract of *Rosmarinus officinalis*, which contains tannins and Saponins was considered, a large variety of microorganisms, including bacteria, are effectively inhibited by these compounds.

Antioxidant activity test: The DPPH radical reduction capacity was measured by decreasing the absorbance at 517 nm caused by antioxidant actions found in both *Rosmarinus officinalis* extracts. According to the results, both extracts can decrease the stable radical DPPH to a yellow color. Concentration (6107.14) produced the highest scavenging activity for both extracts. As seen in Table 4, Alcohol extract > ascorbic acid > aqueous extract.

Table 4: The antioxidant activity of Rosmarinus officinalis aqueous, alcohol and ascorbic acid against DPPH radicals.

Extract	Concentration (ppm)	Scavenging activity %
Aqueous extract	2035.71	65.32
	4071.41	71.42
	6107.14	74.53
Alcohol extract	2035.71	66.49
	4071.41	85.31
	6107.14	95.23
Ascorbic acid	23	80.03

The antioxidant activity of *Rosmarinus officinalis* extracts is related to the antioxidant agents found in them; Redox properties of these antioxidants help in the removal and absorption of free radicals [36]. The results showed that increasing the concentration of each extract enhanced its scavenging activity. In all concentrations, alcohol extract had greater antioxidant activity than aqueous extract; this could be due to alcohol extract's higher flavonoid and phenolic content than aqueous extract [36]. The antioxidant activity of alcohol extract at 6107.14 ppm was found to be greater than that of ascorbic acid at 23 ppm. On the other hand, the aqueous extract's scavenging activity at 6107.14 was less effective than 23 ppm ascorbic acid.

Plant elements with antioxidant activity play an important role in disease prevention and health maintenance [36]. As was previously mentioned, the polyphenols found in rosemary's leaves—primarily rosmarinic acid, carnosic acid and carnosol accumulate in the lipid membranes of cells, where antioxidant activity is needed [45].

Conclusion

As a result of the current work, the Gram-positive bacteria were more common in bacterial conjunctivitis, whereas gram-negative bacteria were less common but more varied. The results also showed that the alcoholic extract of rosemary has good antibacterial, phytochemical, and antioxidant properties. Rosemary extract can be utilized as an antibiotic in pharmaceutical products and medical herbs in the form of ointments or eye drops.

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