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In Vitro Evaluation of *Apocynum cannabinum* Against Different Bacteria

Anjum Masood[1], Tariq Nadeem[2], Muhammad Umair Manzoor[3], Muhammad Owais[3], Muhammad Imran Khan Sohrani[4], Zohaib Saeed[5], Muhammad Rizwan[5], Rustam Ali Balouch[5] and Qazi Muhammad Awais[5]

[1]Department of Theriogenology, University of Agriculture Faisalabad, Pakistan; [2]Para Veterinary Institute Karor Lal Eason Layyah, Sub Campus University of Veterinary and Animal Sciences, Lahore; [3]Wah Medical College, National University of Medical Science, Rawalpindi, Pakistan; [4] Department of Clinical Medicine and Surgery, University of Agriculture Faisalabad; [5]Multan College of Veterinary Sciences, Multan University of Science and Technology, Multan, Pakistan
*Correspondence: zohaibsaeedahmad@gmail.com

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ABSTRACT

This research was conducted to check the antibacterial activity and cytotoxic effects of the extracts of *Apocynum cannabinum L.*, which is commonly called Indian hemp. It is widely known as a medicinal plant due to its ethno-pharmacological applications. The soxhlet apparatus was used to extract the leaves of *A. Cannabinum* with chloroform, hexane, distilled water, and hexane. These extracts were used against four major pathogens of poultry, *Clostridium, E. coli, Salmonella,* and *Staphylococcus*. The zone of inhibition was determined by the agar well diffusion method, while the serial dilution assay was used for minimum inhibitory concentrations. The cytotoxic effects were determined by MTT assay using the Vero cell lines to check the safety of the extracts. The results showed that the ethanolic and chloroform extracts show significant antibacterial effects against the isolated bacteria, while the hexane extracts showed moderate results, and the aqueous extracts showed results only against *Clostridium*. The chloroform extract shows the best results against the bacteria with low MIC values. The results from the MTT assay showed that concentrations of chloroform (3610.74), ethanol (4025), hexane (2850), and aqueous extracts (1561.75) µg/ml have no cytotoxic effects. All the results were statistically observed by using Duncan's Multiple range post hoc test, which showed significant variations in MIC and ZOI values between different groups, whereas no variations were observed in the same group. Overall, results showed that the extracts of *A. Cannabinum* may serve as a safer antibacterial agent against tested bacteria.

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INTRODUCTION

Extensive research has been conducted to explore new antimicrobial agents from sources such as plants, microorganisms, and animals (Abreu et al. 2012). The products derived from plants have been used worldwide to solve a wide range of health issues. Phytochemicals that were extracted from plants typically possess the ability to inhibit or kill bacterial growth. For the development of new drugs, many plants and their therapeutic properties have been studied. In comparison to engineered and synthetic drugs, plant-based antimicrobials have lower adverse effects and have significant therapeutic potential for treating many infectious diseases (AlSheikh et al. 2020). The phenomenon of drug resistance among pathogenic microorganisms has been frequently documented on a global scale. Antibacterial resistance is an increasing issue in the medical discipline. The underdeveloped countries experience a higher rate of resistance to these medications, largely due to the extensive and non-selective use of antibiotics. Moreover, people often take self-medication without proper examination and consultation. The medicinal plants have many valuable compounds that can be administered as therapeutic agents for many health conditions (Sun and Shahrajabian

2023). Plant materials have been an important source for solving many health issues. Many plants have potential new drugs for the creation of innovative medicine. There has been an increase in the emergence of numerous pathogens that have drug resistance, and this has necessitated the need to come up with new antimicrobial substances using alternative sources, such as plants. *Apocynum cannabinum* has been used traditionally in treating some health issues and snake bites (Vásquez et al. 2015). It also portrays hepato-protective effects, is effective in killing the bacteria, and also deals with hypercholesterolemia. *Apocynum cannabinum* belongs to the *Asclepiadaceae* family. *Apocynum cannabinum* is applied as an anodyne, an astringent, one that elevates digestion and appetite, a liver tonic, a cardio tonic, an emetic, a diuretic, an anthelmintic, and an expectorant. It also has some other good effects, like it can be used as a serotonin, it also shows effects in pyrexia, diabetes, and is effective in remedies for weight loss. It has been reported that different aqueous extracts and organic compounds of *Apocynum cannabinum* leaves have antimicrobial effects against a wide range of bacterial pathogens, including some strains of *Bacillus, Pseudomonas, and Staphylococcus* (Jafarı-sales et al. 2020). The poultry industry is in great danger from many microbial organisms. Poultry diseases Salmonella species. The common poultry diseases include enteritis, coryza, septicemia, arthritis, bumble foot, and coccidiosis, which is an intestinal disease (Saeed and Alkheraije 2023). In response to such bacterial infections, antibacterial agents or antibiotics are administered to either treat or prevent these infections. But unfortunately, in our country, the antibacterial resistance is increasing day by day. So there is a need for new antibiotics that show results against antibiotic-resistant bacteria. The primary focus in the search and development of a new antibacterial agent is to ensure maximum safety and minimal lethality (World Health 2020). By considering this aspect, natural sources such as shrubs, herbs, and plants are being explored for their antibacterial activity, as they possess a broader therapeutic index and are safe to use. Consequently, this research aimed to solve the above-mentioned issues and induce new medicines for poultry infections with an emphasis on cost-effectiveness and safety (Bester et al.).

MATERIALS AND METHODS

The Soxhlet apparatus was used to extract the compounds of *Apocynum cannabinum* with distilled water, hexane, ethanol, and chloroform (Abubakar et al. 2022). The well diffusion technique is used to determine the antimicrobial efficacy in relation to the above-mentioned bacteria. MIC (minimum inhibitory concentrations) were calculated on the extracts that showed an antibacterial effect. All the extracts were tested with regard to their cytotoxicity on the Vero cell line via the MTT assay (Dwidhanti et al. 2018). New leaves were obtained and known and verified by the Department of Botany. The ground leaves were used, and 200g was used to prepare the crude extract. To dry up the extracts, we use an incubator and dry the extracts at 37°C and store them in dark airtight containers, and weigh to determine the percentage yield using the following calculation (Mphahlele et al. 2016): Evaluation of antibacterial activity. All bacteria used in the experiment were isolated on specific media and then Gram-stained along with numerous tests, including the IMVIC test and catalase test (Pritom 2022). Preparation of stock solutions of ethanol, chloroform, and hexane was done by making a solution of 1mg extract per 1 mL DMSO. Along with it, the preparation of aqueous extract was done in 1 ml phosphate buffer saline by the addition of 0.1 g of plant extract.

Antibacterial activity

The wells were created, and the petri plates that were used were first sterilized (Peng et al. 2023). Then a sterilized swab, which was dipped in bacterial suspension, was swabbed on petri dishes. Then, 200µl of DMSO was introduced into the control wells, and 200µl of each extract was introduced into the wells. Then these wells were subjected to the incubator for 24 hours, and then the zone of inhibition was measured and further analyzed to determine the Minimum Inhibitory Concentration (MIC) using the serial dilution method. The 96-well ELZA plates were used for the MIC assessment (Elshikh et al. 2016). Now we fill the wells from 1-12 from each row, and we add a volume of 200µl of nutrient broth to them. Following this, 200µl of extracts were introduced into well No. 1 and subsequently diluted two-fold up to well No. 10. Afterward, 200µl was removed from well No. 10 and discarded. This procedure was repeated for the remaining extracts. Next, 200µl of bacterial suspension was added to wells 1 through 11. The same procedure was repeated for all bacterial isolates. All Petri dishes were placed in an incubator with a set temperature of 37°C for 24 hours (Chen et al. 2020). The Dishes were covered, wrapped, and labelled. The ELIZA reader was used to measure the optical density (Abbasi et al. 2021).

Cytotoxicity assay

We conduct MTT Assay and prepare MTT dye in specific concentrations for each dilution and test them individually (Ghasemi et al. 2021). For the test, we made 2 groups: one is the experimental group, and the other is the control group. The control group contained 2 further positive and negative groups. The positive group contained media along with 25% DMSO, and the negative group had only the media. The experimental group contains media, the extracts, and MTT dye (300µg/ml). The concentration of the extract solution 14475, 25050, 16350, and 5750µg/ml of chloroform, aqueous, ethanol, and hexane, respectively, was added to separate wells at 300µg/ml for testing and control. Triplicate wells were used to test each dilution (Keil et al. 2016). The petri dishes were incubated at 37°C for 48 hours. Results were expressed as cell survival percentage (CSP) using the following formula: Statistical analysis: The data collected were statistically evaluated using SPSS version 16.0. The results of the (ZOI) and (MIC) were analyzed using one-way ANOVA, while the antibacterial and MTT assay results were compared using the (DMR) Post-hoc test at $P \leq 0.05$ (Lee et al. 2017).

RESULTS

In this research, the extracts of *Apocynum cannabinum* leaves yielded were recorded as 13.5, 2.1, 1.7, and 11.2% ethanol, chloroform, hexane, and aqueous extracts, respectively, as shown in Table 4. The ethanol yield was recorded as the highest among them, followed by the aqueous extract. Hexane and chloroform show a lower yield. The variation in the yield shows the polarity of solvents and the solubility of the medical content present in the leaves of the plant. The zone of inhibition was measured to determine the antibacterial activity produced by each extract against the mentioned bacteria, as shown in Table 1. The results show that chloroform and ethanol extracts show the most consistent antibacterial activity against all tested strains. The chloroform extract shows marked inhibition against the bacteria, such as Staphylococcus and Clostridium, 12.3 ± 1.5 mm, 18.5 ± 2.1 mm, respectively. Also, there is some inhibition for Salmonella and E.coli. Hexane extract shows strong antibacterial activity against Clostridium, 19.2 ± 2.0 mm, and moderate ZOI for Staphylococcus and for E.coli. Hexane shows limited effects with inhibition only against Clostridium 17.1 ± 1.6 mm. No activity was recorded against E. coli, but there were some effects against Salmonella and Staphylococcus. Aqueous extracts show inhibition only against Clostridium 13.2 ± 1.8 mm. With these results, we can clearly observe that the chloroform and ethanol extracts show consistent antibacterial activity against all the above-mentioned bacteria. Statistical analysis revealed that a significant difference was observed between the inhibition zone diameters (ZOI) and MIC values between extracts versus each other, but there was no significant difference between extracts in each group. These observations indicate that the chloroform fraction had a higher concentration of active antibacterial compounds than the hexane, ethanolic, and aqueous fractions. The aqueous extract was not very active, as it was capable of inhibiting only one bacterium. When we see the antibacterial activity of extracts of A. cannabinum in terms of MIC (minimum inhibitory concentration the chloroform extracts show the highest activity, followed by the hexane extract, as shown in Table 2. The aqueous extract shows activity against one bacterium, Clostridium, and the ethanolic extracts show very minimal activity against the bacteria. When we see all the tested bacterial strains, we observe that only Clostridium is the most susceptible among all the bacteria because it responds positively to all the extracts and shows the highest inhibition zones, which range from 11 to 21mm approximately (Gohari and Prescott 2022). On the other hand, no other tested bacteria show such wide inhibition zones. Statistical analysis revealed that a significant difference was observed between the inhibition zone diameters (ZOI) and MIC values between extracts versus each other, but there was no significant difference between extracts in each group. These observations indicate that the chloroform fraction had a higher concentration of active antibacterial compounds than the hexane, ethanolic, and aqueous fractions. The aqueous extract was not very active, as it was capable of inhibiting only one bacterium. In order to further determine cytotoxicity, an MTT test was conducted on the concentrated solutions of the extracts. All four extracts were not deemed toxic at low concentrations. Nevertheless, cell survival percentages (CSP) were below 50 percent when assayed at high concentrations: 36.8 percent hexane at 5600 µg/ml, 42.5 percent chloroform at 7050 µg/ml, 46.1 percent ethanol at 7980 µg/ml, and 40.3 percent aqueous extract at 3250 µg/ml, as shown in Table 3. Surprisingly, only C. perfringens type A had an M Chloroform extract; however, it lay within the range of safe CSP that applied to all the sensitive bacterial strains, and thus it is the most promising extract. While on the other side, the ethanolic extract shows cytotoxicity on the tested bacterial strains, and the aqueous extract shows very low antibacterial activity against only one bacterium, Clostridium. Collectively, these results show that the chloroform extract of *Apocynum cannabinum* leaves has the highest and safest antibacterial effect against the pathogens tested. The hexane fraction is selectively non-toxic to C. perfringens type A, but the ethanolic and aqueous fractions seem not to be suitable, because of cytotoxic effects. The results of the MTT experiment demonstrate that this plant may be useful in therapy, provided the active antibacterial substances are obtained, purified, and transformed to a stable and safe pharmaceutical form (Karakaş et al. 2017).

DISCUSSION

The research shows the antibacterial activity and cytotoxic effects of extracts of the plant *Apocynum cannabinum* against tested bacterial pathogens (Abubakar et al. 2022). The results show that the chloroform and ethanol extracts show strong antibacterial activity, while the aqueous and hexane extracts show less effect. This study suggests that

Table 1: Zones of inhibition (ZOI in mm) of *Apocynum cannabinum* leaf extracts against poultry bacteria.

Bacteria	Hexane (mm)	Chloroform (mm)	Ethanol (mm)	Aqueous (mm)
Salmonella	7.2 ± 0.6	8.5 ± 0.8	9.1 ± 0.9	0.0 ± 0.0
Staphylococcus	6.4 ± 0.5	12.3 ± 1.5	10.2 ± 1.1	0.0 ± 0.0
Clostridium	17.1 ± 1.6	18.5 ± 2.1	19.2 ± 2.0	13.2 ± 1.8
Escherichia coli	0.0 ± 0.0	9.8 ± 1.0	8.7 ± 0.9	0.0 ± 0.0

Table 2: Minimum inhibitory concentration (MIC, µg/ml) of *Apocynum cannabinum* leaf extracts against poultry bacteria.

Bacteria	Hexane	Chloroform	Ethanol	Aqueous
Salmonella	5600.0 ± 300.0	3610.7 ± 200.0	4025.0 ± 250.0	3250.0 ± 180.0
Staphylococcus	5750.0 ± 200.0	3610.7 ± 150.0	4025.0 ± 300.0	3250.0 ± 200.0
	5750.0 ± 250.0	3610.7 ± 180.0	4025.0 ± 270.0	3250.0 ± 220.0
Clostridium	2850.0 ± 0.0	3610.7 ± 100.0	4025.0 ± 150.0	1561.8 ± 100.0
Escherichia coli	5750.0 ± 250.0	3610.7 ± 200.0	4025.0 ± 300.0	3250.0 ± 180.0

Table 3: Cell survival percentage (CSP% %) of *Apocynum cannabinum* extracts in the Vero cell line (MTT assay).

Extract and conc. (µg/ml)	CSP (%)
Hexane at 5600 µg/ml	42.5
Chloroform at 7050 µg/ml	46.1
Ethanol at 7980 µg/ml	46.1
Aqueous at 3250 µg/ml	40.3

Table 4: Extract yield of *Apocynum cannabinum* leaves.

Extract	Yield (%)
Ethanol	13.5
Chloroform	2.1
Hexane	1.7
Aqueous	11.2

biologically active compounds of A. cannabinum show more effects with chloroform and ethanol as compared to hexane and aqueous extracts (Motiejauskaitė et al. 2023). The inhibitory activity of chloroform extracts against Clostridium and Staphylococcus is important because both bacteria are major bacteria of poultry disease and cause economic loss in the poultry industry. The resistance to antibiotics becomes an increasing challenge, making the research of alternative plant-derived antibiotics highly relevant. Another study in which phytochemical analysis and antimicrobial activity of *Apocynum cannabinum* were carried out revealed that the plant has an abundant amount of saponins and various other phytochemicals, which have therapeutic properties (Abubakar et al. 2022). The Chloroform extract from both the aerial and root parts of *Apocynum cannabinum* showed greater antimicrobial activity as compared to diethyl ether and acetone. The chloroform root extracts showed competitive minimum inhibitory concentration (MIC) and minimum bactericidal concentration values against the pathogens. These findings corroborate the outcomes of the current study, as the chloroform extract showed antibacterial properties against all experimental bacterial strains. A comparable investigation was performed on essential oils derived from *Apocynum cannabinum* leaves, which demonstrated an inhibitory effect on the growth of some strains of Pseudomonas, Bacillus, and Escherichia coli (Abubakar et al. 2022). The present results are in line with those of this research because Escherichia coli, a widespread pathogen in the two studies, was sensitive to both the chloroformic and ethanolic extracts of *Apocynum cannabinum*. Also, the *Apocynum cannabinum* leaves aqueous and methanolic extracts showed moderate activity on the three pathogenic Salmonella species (S. typhi, S. typhimurium, and S. paratyphi). The aqueous extract was more efficient on the Salmonella species compared to the other extract (aqueous). This paper supports the antimicrobial effect embedded in the aqueous extract of *Apocynum cannabinum*. The extract of *Apocynum cannabinum* leaf in ethanol exhibited a great antimicrobial effect on some bacteria, such as Staphylococcus and Pseudomonas. E. coli responded to chloroform and ethanolic extracts, which contradicts the earlier findings (Imran et al. 2021). Aqueous and Methanol extracts of *Apocynum cannabinum* leaves had a moderate effect on some pathogenic species of Salmonella (Chomini et al. 2021). The aqueous extract was more active than the other two extracts that are used against the Salmonella species. The prepared extracts using the successive solvent extraction methods were tested based on their antimicrobial effects against some strains of Streptococcus and Candida albicans using the Agar well diffusion method with doses of 50 and 100 mg/ml. The zone of inhibition (ZOI) was 12-23 mm against the 25 mg/ml of the methanol extract, and it showed a lot of antimicrobial effects (Begashaw et al. 2017).

In addition, the results of the current research support the existence of chemical compounds that have antibacterial effects in *Apocynum cannabinum* (Abubakar et al. 2022). These findings were useful in giving insights into the antibacterial action of *Apocynum cannabinum* leaves and, in addition, justify the use of the plant in the field of traditional medicine practices. Although they used the disc diffusion method instead of the well diffusion method, they got the same results as the current literature, implying that bacteria were more vulnerable to the chloroform extract and least vulnerable to the aqueous extract of *Apocynum cannabinum*. The anti-bacterial activity of the *Apocynum cannabinum* leaves was tested against four Gram-negative (E. coli, Pseudomonas, Staphylococcus, and Salmonella) and three Gram-positive bacteria (Bacillus, Staphylococcus, and Micrococcus luteus) in different solvents (Bushra et al. 2025). The results showed that the extracts of all the used solvents had good effects against the mentioned bacteria. The increase in antibacterial activity was found to increase with an increase in the extract concentrations (Elisha et al. 2017). No antibacterial effects were recorded at concentrations of 10 mg/ml and 20 mg/ml. The current studies show that chloroform and ethanolic extracts (the common solvents in both studies) exhibited notable antibacterial activity against various Gram-positive and Gram-negative microorganisms. According to the findings of the zone of inhibition (ZOI), E. coli was observed to be the most sensitive type of microorganism, whereas Pseudomonas was observed to be the resistant type of microorganism. The findings confirm the comprehensive activity of *Apocynum cannabinum*, indicating its possible application in the preparation of new antimicrobial agents. The solvents used in the present research are different from those used in the earlier research, but the similarity is that in both studies, the bacteria were susceptible to the extract of *Apocynum cannabinum*, thus enhancing the fact that this plant does have the potential to produce antibacterial activity. Aerial extracts of *Apocynum cannabinum* (ethyl acidic, chloroform, ethanol extracts) showed an antibacterial effect on Escherichia coli, Pseudomonas, Klebsiella, and Staphylococcus (Latacumba and Esteban 2020). The current research has revealed that chloroformic and ethanolic extracts had the highest antibacterial effects, followed by hexane, and the aqueous extract had the weakest effect. Chloroform and ethanol were found to be the best solvents in the extraction of a vast array of antimicrobial compounds in *Apocynum cannabinum*. The current research revealed that the relationship between zones of inhibition (ZOI) and minimum inhibitory concentration (MIC) values of crude extracts from *Apocynum cannabinum* leaves varies against different experimental bacterial strains (Abubakar et al. 2022)

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