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Biological Activities of Polar Extracts and Phytochemicals Isolated from *Trifolium Hybridum* L.

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Abstract: *Trifolium hybridum* L. (Alsike clover), a member of Fabaceae, is mainly used as a fodder crop. However, the related species such as *T. repenes, T. arvense, and T. pratense* have already been explored for their antioxidant, antiseptic, and anti-inflammatory potentials. As little information was available on *T. hybridium*, the current study aimed to monitor the pharmaceutical role of root, stem, and leaves extracts of *T. hybridum* on microbial infections and oxidative stresses via, *in vitro* approach. The study revealed that ethanolic extract of leaves showed the highest inhibitory potential of 24.6 ± 0.57 mm against *K. pneumoniae* and acetone extract of leaves showed the highest inhibition of 22.3 ± 1.15 mm against *Morganella*. Other extracts including ethanol and acetone showed moderate inhibition against other selected microbes including methacillin-resistant S. *aureus*. Regarding the antioxidant activity tannins extracted from leaves showed higher activity with an absorbance of 1.951 ± 0.096 compared to flavonoids, alkaloids, and β-carotenes. Furthermore, compound isolation and purification showed a total of 20 fractions, which were further evaluated in antimicrobial assays. All were proved to be less active as compared to crude extract. Therefore, the crude extract of *T. hybridum* was considered more potent and suggested for further studies for isolation and identification of potential novel compounds.

Keywords: *Trifolium*, phytochemicals, chromatography, medicinal plants.

从三叶草中分离的极性提取物和植物化学物质的生物活性

摘要：三叶草（阿尔斯科三叶草),豆科植物，主要用来作饲料作物。然而，相关物种如三叶草、三叶草和 T.红三叶草的抗氧化、防腐和抗炎潜力已被探索。由于有关三叶草杂交种的信息很少，目前的研究旨在通过体外方法监测三叶草杂交种的根、茎和叶提取物对微生物感染和氧化应激的药用作用。研究表明，叶子的乙醇提取物对肺炎克雷伯菌的抑制潜力最高 24.6 ± 0.57 毫米,叶子的丙酮提取物对摩根氏菌的抑制潜力最高 22.3 ± 1.15 毫米。其他提取物（包括乙醇和丙酮）对其他选定的微生物（包括耐甲氧西林金黄色葡萄球菌）表现出中等程度的抑制作用。关于抗氧化活性，与类黄酮、生物碱和 β-胡萝卜素相比，从叶子中提取的单宁显示出更高的活性，吸光度为 1.951 ± 0.096。此外，化合物分离和纯化显示总共20个级分，并在抗菌测定中进一步评估。与粗提物相比，所有这些都被证明活性较低。因此，三叶草杂交种的粗提物被认为更有效，并建议进一步研究潜在的新化合物的分离和鉴定.

关键词：三叶草，植物化学物质，色谱，药用植物.

Abbreviations

T. hybridium - *Trifolium hybridium*, T. repenes - *Trifolium repenes*, *K. pneumonia* - *Klebsiella pneumonia*, DMSO - dimethyl sulfoxide, *H. influenza* - *Haemophilus influenza*, L.B - Lauria-Bertini.

1. Introduction

Medicinal plants are vital sources for treating several fatal diseases [1]. Traditional medicinal approaches have mainly used medicinal plants for the treatment of various diseases since the beginning of human civilization and still play an important role in covering the basic health needs in developing countries. The significance of these medicinal plants is due to the presence of various classes of phytochemicals that trigger a distinct physiological effect on the human body [2, 3]. Similarly, the extracts of several plants also possess some novel supplies of antibacterial potential with possibly new mechanisms of action [4, 5]. These phytochemicals are helpful in the curing of infectious disorders while concurrently justifying the side effects linked with synthetic antibacterial agents [6]. The pharmaceutical industry faces serious challenges in drug discovery because it is costly, critical, inefficient and risky [7, 8]. The minimum cost of new drug discovery is about 1 billion USD in 12 years. A hope of new chemical entities is provided by the medicinal and combinatorial chemistry that succeeds in developing many chemical libraries. However, in terms of overall success, these approaches have many limitations [9, 10].

There are several biomolecules isolated from natural sources that are effective against different diseases, including inflammation, neuropharmacological, gastrointestinal, cardiovascular, metabolic disorders, cancer, and other infectious diseases [11]. According to the World Health Organization (WHO), more than 80% of the population in the world relies on traditional herbal medicine for their primary healthcare [12]. There is a long history of using plants for chronic and infectious diseases in Asia [13]. The increasing antimicrobial resistance and adverse effects of synthetic drugs have compelled men to turn back to ethnopharmacognosy [14]. Recently, scientists across the globe are now trying to find less adverse and broadly effective alternatives. The traditional claims of biologically active compounds can only be verified by their bioavailability, safety, efficacy, pharmacokinetics, and pharmacodynamics [15]. According to WHO, there are about 20,000 medicinal plant species in 91 countries including Pakistan and 85,000 medicinal plant species in all other countries throughout the world. Pakistan has a diverse climatic situation that supports the development of nearly 700 plant species reported for their medicinal value, and the number is rising continuously due to the present attention of local researchers in natural products [16].

Leguminoseae or Fabaceae account for a considerable number of medicinal plants due to the presence of some precious phytochemicals. This family is also known as pea or bean family and comprises economically and medicinally important herbs, shrubs, trees, annual or biennial flowering plants, which can easily be recognized by their stipulated leaves and legume fruits [17]. This family is found all over Europe, Asia, and the USA. The morphological properties and chemovariability of the genus *Trifolium* belonging to the same family have attracted great interest of the scientific community due to its pharmacological importance [18]. The herb *Trifolium hybridum* belongs to the genus Trifolium of this family. The flowers are stalked, bisexual, and unmarked trifoliate leaves. The best flowering season is from June to September. The height of the plant is about 1-2 feet (30-60 cm). The Turks and

many other societies are using different species of *Trifolium* such as *T. pratense*, *T. arvense* and *T. repens* due to their pharmaceutical importance such as antiseptic, antirheumatic, antioxidant, anti-inflammatory and antidiabetic properties but till now the therapeutic properties of *T. hybridum* are explored to very less extent [19]. About 60% of Pakistani populations are still using medicinal plants for the treatment of different diseases [12]. The main reason behind this is that the whole of Pakistan and specifically Khyber Pakhtunkhwa is very rich in medicinal plants, but unfortunately, they are not systematically examined for their precious biological activities [20]. On the basis of the above-mentioned facts, the current study was carried out to assess the antioxidant potential and antimicrobial properties of *T. hybridum* against selected resistant pathogens as Trifolium species are proven to be rich sources for phytoactive compounds. This will later help in the development of drug that would be potent against infectious microbes and further reduce the oxidative stress generated by microbes.

The evaluation of antioxidant potential and antibacterial properties of Trifolium hybridium, a member of the Leguminoseae or Fabaceae family, is what distinguishes this work. While other Trifolium species have been studied for their medicinal properties, the therapeutic potential of *T. hybridium* has only been explored to a limited extent. This study seeks to fill this knowledge gap by investigating *T. hybridium*'s possible medical benefits.

T. hybridum was selected because compounds found in *T. hybridum* have been demonstrated to be effective for treating a variety of health issues including cancer, inflammation, neurological disorders, gastrointestinal difficulties, cardiovascular problems, metabolic disorders and infectious diseases.

2. Methods

2.1. Determination of Bioactive Compounds

In the first series of experiments, various secondary metabolites including flavonoids, tannins, alkaloids, β-carotene and lycopene were extracted from roots, stems, and leaves of *T. hybridum* (Fig. 1).

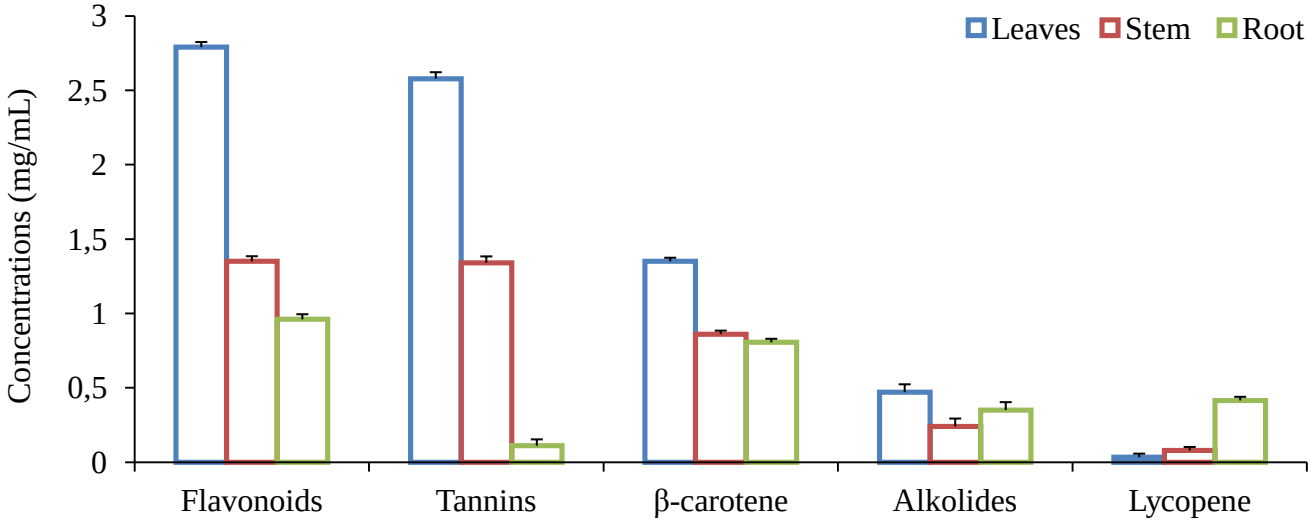

Fig. 1 The figure shows the concentrations of flavonoids, tannins, β-carotene, alkaloids, and lycopene in the leaves, stem, and root extracts of *T. hybridium.* All values were measured in triplicate.

The absorbance obtained for flavonoids in the leaves was 2.791 ± 0.096 at 250 µl while that for stem and root parts was 1.355 ± 0.329 and 0.963 ± 0.029, respectively, at 398 and 275 µg/mL. Similarly, the tannin contents in the leaves were higher in concentration (2.572 ± 0.0372) at 250 µl, while the stem and roots were noted to contain 1.348 ± 0.0338 and 0.1137 ± 0.0313 mg/mL, respectively. Results reveal that the leaves of *T. hybridum* contain the highest concentration of alkaloids (0.47 ± 0.03 mg/ml) as compared to stem and root, which were found 0.24 ± 0.04 mg/ml and 0.35 ± 0.06, respectively. Similarly, the β-carotene are found higher in leaves (1.351 ± 0.010 mg/50 ml) then stem (0.868 ± 0.0005 mg/50 ml) and root parts (0.805 ± 0.030 mg/50 ml). The concentration of lycopene in leaves of *T. hybridium* is 0.0333 ± 0.001 mg/50 ml while stem and roots contain 0.078 ± 0.0005 mg/50 ml and 0.415 ± 0.039 mg/50 ml respectively. This study revealed that compared to other parts, *Trifolium* leaves contained significant concentrations of flavonoids, tannins, β-carotene, alkaloids, and tannins. Our results agree with the findings of Esmaeili [21], who showed that *T. partenes* leaves also contain a high amount of flavonoids compared to other parts of the plant [24].

2.2. Antioxidant Activities of Flavonoids and Tannins

Antioxidant activities of the extracted flavonoids and tannins from the root, stem, and leaves of *T. hybridum* were determined using a free radical scavenging assay (Fig. 2). The concentrations of flavonoids and tannins in the leaves were 0.71 ± 0.0351 and 1.91 ± 0.0264

mg/mL. Similarly, flavonoids and tannins from the stem (0.47 ± 0.0291 and 1.12 ± 0.0201 mg/mL) and root also possess some considerable antioxidant potential (0.47 ± 0.0152 and 0.246 ± 0.0193 mg/mL).

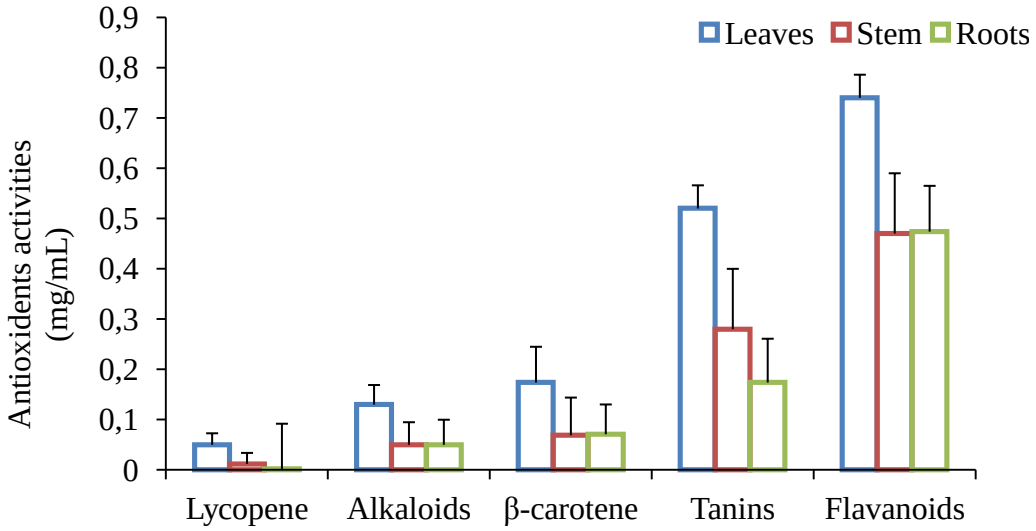

Fig. 2 The antioxidant activities of flavonoids and tannins in leaves, stems and roots of *T. hybridium*. All values were measured in triplicate.

2.3. The Antioxidant Activity of Different Solvent Extracts

In vitro antioxidant activity of different solvent extracts, i.e., n-hexane, acetone, chloroform, butanol, ethanol, methanol and distilled water from leaves, stem and roots of *T. hybridum* was assessed in comparison to that of standard ascorbic acid (Fig. 3). Among all, methanol and ethanol based extracts have shown the highest antioxidant activities, n-hexane, acetone and chloroform extracts have shown moderate activities while butanol and aqueous extracts showed negligible activities. The results of individual solvent extracts from different parts (leaves, stem, and root) showed diverse antioxidant activities. However, among all, the methanol extract of leave was found significant (1.413 ± 0.099 mg/mL) as compared to stem and root, i.e., 1.294 ± 0.051 and 1.139 ± 0.061 mg/mL, respectively, at 750 µg. Likewise, the antioxidant activity of the ethanol extract was found to be maximum in the leaves, with 1.317 ± 0.074 mg/mL at 7500 µg. While the n-hexane, i.e., 0.791 ± 0.91, 0.732 ± 0.053 and 0.913 ± 0.039 mg/mL were found for the leaves, stem and roots respectively. Similarly, in acetone, chloroform, butanol and water the leaves contain maximum antioxidant capabilities i.e., 0.616 ± 0.088, 0.712 ± 0.091, 0.437 ± 0.061, and 0.479 ± 0.088 mg/mL respectively as compared to standard ascorbic acid.

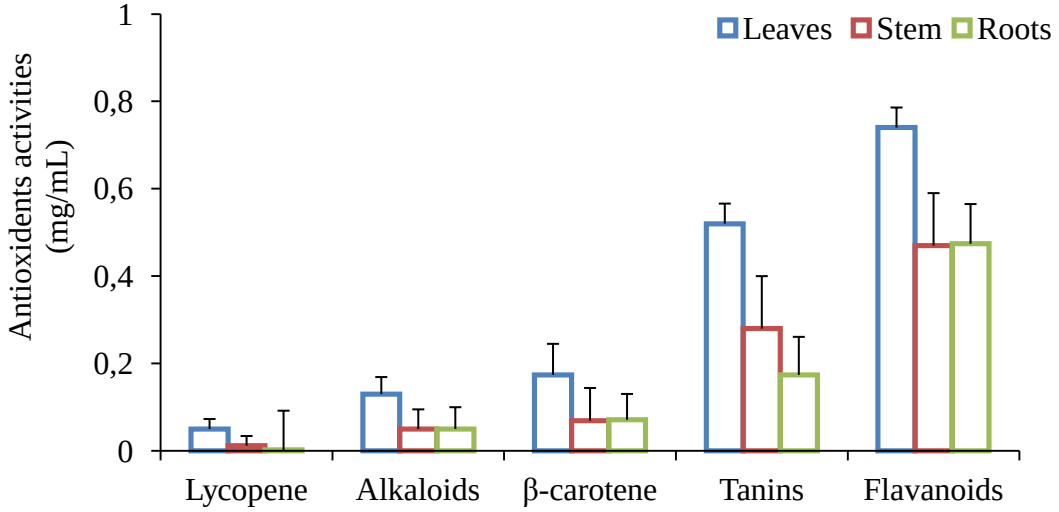

Fig. 3 Antioxidant activities of different solvent extracts of *T. hybridium* parts. Bars show antioxidant activities of the solvents with their respective plant parts. The mean was taken in triplicates.

2.4. Antimicrobial Activities of Different Extracts

In the present study, the antibacterial activity of solvent extracts including n-hexane of roots, stem and leaves of *T. hybridum* was evaluated against selected bacterial strains (Fig. 4A). The results indicated that leaves extracts possess a significant antibacterial potential against *S. aureus*, showing 21 ± 1.52-mm inhibition

Among all extracts, the leaves acetone extract was found very active against *M. morganii* showing an

inhibitory zone of 22.3 ± 1.15 mm, as compared to standards i.e. ampicillin and streptomycin (Fig. 4B). Similarly, its activity against *A. baumanii, K. pneumoniae* and *P. aeruginosa* was noted with inhibitory zone of 17.6 ± 1.15, 17.3 ± 1.52 and 15 ± 2 mm, respectively, while no significant effect against *E. coli* was observed. In the case of stem, its acetone extract showed a high inhibitory potential against *P.aeruginosa*, i.e. 19 ± 1 mm. However, the root acetone extract had a good antibacterial activity against *A. baumannii* and *K. pneumoniae* but showed moderate activity against other bacterial pathogens.

The results indicated that the chloroform extract of leaves possessed high inhibitory potential against *A. baumannii* with a zone of inhibition of 17.6 ± 1.5 mm, while showing 14.67 ± 1.15, 13.6 ± 0.5 and 14.3 ± 1.15 mm zone of inhibition against *M. morganii, P. aeruginosa* and *K. pneumoniae*, respectively. The chloroform extract of the stem and root did not show any satisfactory results against all the selected bacteria (Fig. 4C).

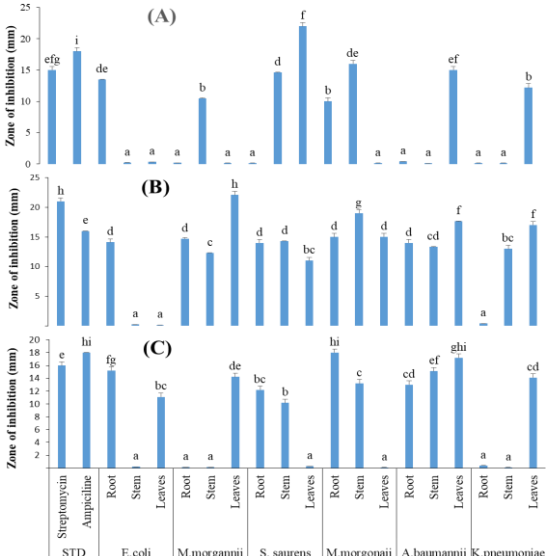

Fig. 4 Different extracts, (a) chloroform, (b) acetone, and (c) chloroform, from the *T. hybridum* roots, leaves, and stem show significant antibacterial activities against different bacterial strains. Various statistical bars represent significant effect compared to slandered at p > 0.05.

The results revealed that the leaves butanol extract showed high inhibitory potential against selected bacterial strains (Figure. 5A). Among all, *K. pneumoniae* had an inhibition zone of 18.3 ± 1.5 mm as shown in the figure, while their stem extract showed 12.13 ± 0.67 and 13 ± 1 mm inhibition against *P. aeruginosa* and *A. baumanii*, respectively, and 15.6 ± 0.57 mm against *E. coli* for root extract.

Ethanolic extracts of leaves show a high inhibitory zone of 24.6 ± 0.57 mm and 23.6 ± 1.52 mm against *K. pneumoniae* and *P. aeruginosa*, respectively, which is higher than the standard antibiotics used against these bacteria (Fig. 5B). Their stem extract shows high inhibitory potential against *P. aeruginosa* with a zone of inhibition of 26.3 ± 1.15 mm, which is even higher than the standard drugs used. Here the extract from root did not show efficient antibacterial activity.

According to the results, the methanol extracts of leaves possess moderate inhibitory potential against *M. morganii, A. baumannii,* and *K. pneumoniae* showing 17 ± 1 mm, 15.6 ± 1.15 mm and 16.3 ± 1.15 mm zones of inhibition, respectively (Fig. 5C). The stem part is effective only against *M. morganii, A. baumannii,* and *K. pneumoniae*. The methanolic extract of roots is also effective against all the selected bacterial strains and shows high inhibitory potential against *E. coli* and *P. aeruginosa* showing 23 ± 0.5 mm and 20.3 ± 0.57 mm zones of inhibition, respectively.

The distilled water extract of leaves showed moderate inhibitory potential against *K. pneumoniae* and *M. morganii* with 16 ± 1.41 mm and 15.5 ± 0.7 mm zones of inhibition, respectively (Fig. 5D). The distilled water extract of the stem showed moderate inhibitory potential against *A. baumannii* and *K. pneumoniae* thus showing 17 ± 1 mm and 16.5 ± 0.71 mm zone of inhibition, respectively. The distilled water extracts of roots are moderately effective against *E. coli* and *P. aeruginosa* showing 17.3 ± 1.15 mm and 15.3 ± 0.57 mm zone of inhibition, respectively, and become least effective against *M. morganii, A. baumannii* and *K. pneumoniae*. The antibacterial activity of different solvent extracts (n-hexane, acetone, chloroform, ethanol, methanol and water) of *T. hybridum* against selected pathogenic bacterial strains i.e. *E. coli, S. aureus, K. pneumoniae, A. baumanii, P. aeruginosa* and *M. morgannii* was evaluated.

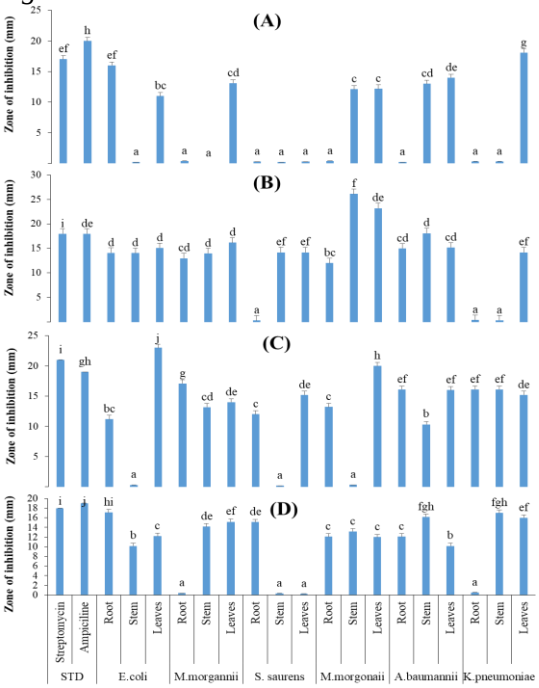

Fig. 5 The antibacterial activities of *Trifolium hybridum* different extracts butanol (A), ethanol (B), methanol (C) and distilled water (D) were monitored against different pathogenic bacteria. Different statistical bars represent significant zone of inhibition as compared to standards used at a level of p > 0.05.

2.5. The Antibacterial Activity of Isolated Phytochemicals

The ethanol extract of leaves showed a high inhibitory potential against *K. pneumoniae* and *P. aeruginosa* showing 24.6 ± 0.57 and 23.6 ± 1.52 mm inhibitory zones, respectively. This might be the presence of high concentrations of phytochemicals, e.g., flavonoids and their derivatives, in the leaves of *T. hybridium*. However, the high inhibitory activities might also be due to the interaction of these active compounds with the outer membrane of bacterial cells, e.g., gram positive and negative bacterial strains might be indicative of the presence of compounds as broad-spectrum antibiotic activities [22].

Flavonoids in leaves revealed high inhibitory activity against *E. coli* and *K. pneumonia* showing 21.3 ± 1.15 and 16 ± 1 mm zones of inhibition, respectively. Similarly, the roots extract significantly inhibited the growth of *M. morganii* and *E. coli* with 19.3 ± 1.15 and 15.3 ± 0.57 mm zone of inhibition, while flavonoids present in the stem showed high inhibitory potential of 20.6 ± 0.57 mm zone of inhibition against *E. coli* (Table 1).

The antibacterial activity of tannins in different parts of *T. hybridum* showed a high inhibitory potential of 20.3 ± 0.57 mm against *S. aureus*, while from leaves it showed a moderate inhibitory potential 15 ± 0.75 mm against *E. coli*. The tannin extract of roots showed moderate inhibitory potential of 15 ± 1 and 14.6 ± 0.57 mm against *M. morganii* and *P. aeruginosa*, respectively.

Therefore, maximum antioxidant activity was found in acetone extract. Similarly, the bioactive compound found in higher concentration after tannins was flavonoids that were extracted in ethanol [23]. Likewise, the results of antimicrobial activity revealed that again acetone and ethanol extracts gave maximum activity as found in case of antioxidant activity. The maximum number of tannins and flavonoids are extracted in these two solvents. Acetone extract of leaves showed its highest inhibitory potential against *M. morgannii A. baumannii* and *K. pneumonia* showing 22.3 ± 1.15 mm, 17.6 ± 1.15 mm and 17.3 ± 1.52 mm zone of inhibition, while the other three microbes, i.e., *E. coli, S. aureus* and *P. aeruginosa* showed resistance against acetone extract.

Table 1 The antibacterial potential of different phytochemicals isolated from *Trifolium hybridum* L.

		S. aureus	***E. coli***	***K. pneumoniae***	***A. baumannii***	***P. aeruginosa***	***M. morganii***
	Standards	G + ve	G –ve	G –ve	G –ve	G –ve	G –ve
Standard	Streptomycin	20 mm	15 mm	16 mm	15 mm	_	_
	Ampiciline	_	18 mm	_	20 mm	18 mm	_
Flavonoids	THL	20 ± 0.5	21.3 ± 1.15	20.3 ± 1.52	13 ± 1	13.6 ± 1.52	16 ± 1
	THS	10.6 ± 0.57	20.6 ± 0.57	13.3 ± 1.52	12.6 ± 1.52	12.3 ± 1.15	10.5 ± 0.7
	THR	_	15.3 ± 0.57	12 ± 0.5	14 ± 0.5	14.6 ± 0.57	19.3 ± 1.15
Tannins	Leaves	20.3 ± 0.57	15.3 ± 0.57	14 ± 0.5	13.6 ± 0.57	17 ± 0.5	12.3 ± 0.57
	Stem	11 ± 1	15 ± 0.75	10.6 ± 0.57	-	11.3 ± 0.57	-
	Roots	12.3 ± 0.57	13.6 ± 0.57	11 ± 0.5	11.6 ± 0.57	14.6 ± 0.57	15 ± 1
Alkaloids	Roots	14.6 ± 1.15	12 ± 1	14 ± 0.5	-	14.6 ± 0.57	-
	Stem	-	10.6 ± 0.57	11 ± 0.57	-	14.6 ± 1.52	11 ± 1
	Leaves	-	13.6 ± 0.57	19 ± 1	12 ± 0.5	-	-
β-carotene & lycopene	Roots	15.6 ± 0.57	12.3 ± 0.57	13.3 ± 1.15	15 ± 0.5	15.6 ± 0.57	12 ± 0.5
	Stem	-	0.9 ± 0.5	10 ± 0.5	-	15.3 ± 0.57	-
	Leaves	_	13 ± 1	23 ± 1	22.6 ± 1.72	18 ± 1	22 ± 1

Note: _ represents unreported.

2.6. Compound Isolation and Purification from Active Extract by Column Chromatography

The crude ethanol extract was prepared and fractionated into 4 fractions, namely, n-hexane, ethyl acetate, di-chloro methane (DCM) and distilled water. The antibacterial activities of these fractions were evaluated to select the active fraction for compound isolation and purification. The results of the antimicrobial activity of these fractions against various selected bacterial strains (Table 2).

Results revealed that among these four fractions ethyl acetate fraction showed highest activity against methicillin-resistant *S. aureus* and *H. influenza* with moderate inhibitory potential of 15 ± 2 and 16 ± 01 mm zone of inhibition respectively as compared to other fractions and is thus selected for further separation via

column chromatography in order to separate the active compound.

Hence 40 different fractions were obtained on different polarities from column chromatography of ethyl acetate fraction and evaluated for antimicrobial potential, among which only 10 fractions showed moderate activity as compared to crude extract (Table 3).

Table 3 The antibacterial activities of different fractions isolated *via*; column chromatography

S. No.	Polarity	*MR S. aureus*	*M. morganii*	*E. coli*	*K. pneumoniae*
1	20% Ethyl acetate	15 ± 0.5	13 ± 1	-	14 ± 0.5
2	25% Ethyl acetate	11 ± 1	-	11 ± 0.5	13 ± 0.5
3	30% Ethyl acetate	-	15 ± 0.5	13 ± 0.5	10 ± 1
4	35% Ethyl acetate	-	-	15 ± 1	-
5	45% Ethyl acetate	12 ± 1	12 ± 0.5	-	-
6	65% Ethyl acetate	-	16 ± 0.5	13 ± 0.5	-
7	70% Ethyl acetate	12 ± 0.5	-	-	12 ± 0.5
8	80% Ethyl acetate	-	-	-	13 ± 0.5
9	85% Ethyl acetate	-	-	10 ± 0.1	11 ± 0.1
10	90% Ethyl acetate	-	15 ± 1	-	14 ± 0.5

Note: - represents no activity.

3. Literature Review

Researchers have been studying the biological properties of medicinal plants in the hopes of discovering a medication for disease treatment and a way to delay the onset of aging symptoms. For this purpose, different secondary metabolites such as flavonoids, tannins, alkaloids, β-carotene and lycopene were studied from roots, stem and leaves of *T. hybridium*. It was found that leaves have the highest concentration of alkaloids and β-carotene then in stem and root, whereas lycopene concentration was found to be lower in leaves than roots. Similarly, each plant family, genus, and species develops its unique chemical properties, which can sometimes be deployed as taxonomic features in the plant classification [12]. It has been assumed that the occurrence of secondary metabolites in plants is extremely difficult to study due to their diversity in phytochemicals and in different classes of plants as well [12]. This study revealed that compared to other parts, *Trifolium* leaves contained significant concentrations of flavonoids, tannins, β-carotene, alkaloids, and tannins. Our findings are consistent with those of Esmaeili et al. [21], who showed that *T. pratense* leaves also contain a high amount of flavonoids as compared to other parts of the plant [21, 24]. By analyzing the antioxidants, it was found that as compared to stem and roots, the maximum antioxidant potential of phytochemicals from leaves was taken into consideration, which could lead to the occurrence of more quantity of these phytochemicals and their derivatives [25]. The results also suggested strong antioxidant potential of *T. hybridum* due the presence of polyphenols in the form of flavonoids and tannins, which are directly involved in the free radicle scavengers and efficient in the stoppage of different oxidative stresses [12, 26]. Among all solvent based extracts, the methanol and ethanol-based extract have revealed the good antioxidant activities, followed by n-hexane, acetone and chloroform extracts with moderate activities, while butanol and aqueous extract showed negligible activities. The methanol extract from leaves revealed the significant antioxidant activity than that of stem and roots. However, antioxidant profiling and phytochemical determination of *T. pratense* and *T. rapens.* The leaves of both species have higher contents of tannins and other secondary metabolites [27]. The present findings are consistent with the study on *T. nigresces* and *T. constantinopolitanum* in which tannins in leaves extract also showed high antioxidant activity [28]. The leaves extract of *trifolium* genus generally contains important phytochemicals, i.e. tannins that have shown significant antioxidant potential. These capabilities might be due to the fact that natural anti-oxidant compounds engage in interactions with free radical assembly and inactivate them by reducing singlet and triplet oxygen, decomposing hydrogen peroxide and inhibiting enzymes per oxidation [29]. Similarly, tannins and flavonoids interact with free radicals and block their chain reactions by donating electrons, reducing and inhibiting their oxidative reactions by oxidizing the reduction reaction [30, 31]. The secondary metabolites and extract of *T. hybridum* isolate from leaves were found to have good antimicrobial activity against selected human pathogens. The acetone, chloroform, butanol and ethanol extracts of leaves showed significant antimicrobial activity.

Similarly, E. crassipes leaf extracts revealed antimicrobial activity against the following bacteria: *E. coli, Bacillus subtilis, Bacillus cereus, Lactobacillus casei* and *Pseudomonas aeruginosa*. As antibacterial and antifungal drugs, streptomycin and fluconazole were utilized as controls. Plant extracts were most effective against B. subtilis, F. solani, C. gloeosporioides, and A. alternate. They used acetone, ethanol, methanol, and n-butyl alcohol to identify the chemical analyzes of the leaf extracts. Several therapeutic components were found in the fractionated extracts, including phenols, proteins, alkaloids, amino acids, polysaccharides, flavonoids, glycosides, tannins,

and terpenoids [32]. The phytochemical analysis and antibacterial activity of several cynodon dactylon extracts (l.) pers. (bermuda) was found that ethanol and ethyl acetate extracts showed the highest activity for all of the tested pathogens. Both methanol and acetone extracts showed activity toward *B. cereus* and *B. subtilis*, while chloroform extract showed activity toward B. subtilis and S. pyogenes, respectively. Diethyl ether extraction showed activity only on S. pyogenes, whereas no activity was observed for n-pentane extraction [33]. In the present study, the ethanol extract of leaves showed a high inhibitory potential against *K. pneumoniae* and *P. auruginosa*. Furthermore, the flavonoids and tannins showed significant antimicrobial activity, which is similar to the findings of [34, 35, 36]. The higher concentration of tannins in leaves might be responsible for the inhibitory effect on these bacterial strains [35].

4. Results and Discussion

4.1. Sample Collection and Preparation

The plant samples of T. hybridium were collected from District Upper Dir, Pakistan for phytochemical determination and evaluation of antioxidant and antimicrobial activities. The entire *T. hybridum* plant was washed and air-dried. Roots, stem and leaf were separated and ground to a fine powder with the help of an electric blender, then saved in fine plastic bags labeled with the name of the respective part and stored for further analysis.

4.2. Phytochemical Determination in Trifolium Hybridum

4.2.1. Determination of Flavonoids

The flavonoid content of a sample was analyzed by dissolving 5 gm in 50 mL of 80% aqueous ethanol and incubated using a shaker incubator for 24 hours. The extract was subsequently filtered and stored at 4 °C in a 50 mL falcon tubes. Spectrophotometry was used to evaluate accordingly. The extract (250 µl) was diluted with 1.25 mL of distilled water and 75 µl of 5% NaNO2 solution. After 5 minutes, 150 µl of 10% AlCl3.H2O was added, followed by the addition of 500 µl of 1 M NaOH and 275 µl of distilled water after 6 minutes. In the same manner, 80% aqueous ethanol blank was developed. The solution was thoroughly mixed, and absorbance at 415 nm was taken for measurement [37]. Different concentrations of quercetin (5 mg-0.3125 mg) were used as standard to draw the standard curve.

4.2.2. Determination of Tannins

Tannins were extracted by dissolving 0.5 gm of sample in 100 mL of 70% Acetone. Estimation of tannins was carried out by a spectrophotometric assay as described by Akindahunsi & Oyetayo [38]. Different concentrations of tannic acid (50 mg-3.125 mg) were prepared by serial dilution from stock solution (50 mg/100 mL of 70% acetone). The absorbance was measured at 725 nm after the addition of 0.5 ml of folin-phenol reagent and 2.5 ml of 20% Na_2CO_3. Blank containing 70% acetone was also prepared in the same way.

4.2.3. Determination of Alkaloids

Alkaloids were extracted using the acid base shifting method described by [39]. According to the protocol, the dried sample was dissolved in ethanol (1:10) and left to shake for 24 hours. Extract was filtered and concentrated near to dryness in an oven and was re-dissolved in ethanol with the addition of 1% HCl. The mixture was placed in a refrigerator for three days. The solution was filtered, pH maintained at 8-10 and extracted with chloroform using a separating funnel. The chloroform layer was recovered and the ethanol layer discarded, while the solution was heated in a hot water bath for evaporation. After that, the sample was dried in an oven to constant weight. Alkaloid contents were calculated on the basis of the weight obtained and weight used.

4.2.4. Determination of β-Carotene and Lycopene

Methanol extract of sample was prepared by dissolving 10 gm of sample in 100 mL (1:10) of methanol and the solution was incubated using shaker incubator at 25ºC for 24 hours. The extract was stored after filtering. The dried sample was extracted with acetone after the liquid extract had been heated in a water bath at 50°C to allow the solvent to evaporate: combination of n-hexane (4:6). The reaction mixture containing ß Carotene and Lycopene was saved at 4ºC. The spectrophotometric analysis was accomplished by measuring the absorbance at 453, 505, 645 and 663 nm. Ss-carotene's contents and lycopene were calculated using the following equations:

ß-carotene (mg/50 mL) = $0.216\ A_{663} - 0.304A_{505} + 0.452\ A_{453}$ (1)

Lycopene (mg/50 mL) = $-0.0458A_{663} + 0.372A505 - 0.0806A_{453}$ (2)

4.3. Bioassays

4.3.1. Extract Preparation for Antimicrobial Activity

The powdered material was extracted using several solvents based on their polarity (n-hexane, Acetone, Chloroform, Butanol, Ethanol, Methanol, and Water). The sample was initially extracted by shaking in n-hexane (1:10) for 24 hours and then filtered. The filtrate was then transferred to a falcon tube that had been pre-weighed, and the residue was re-extracted using a solvent slightly more polar than n-hexane. The

same procedure was repeated with all solvents and all extracts were allowed to dry in an oven at 50°C, and again dissolved in dimethylsulfoxide (DMSO) for antimicrobial assay to obtain a final concentration of 15 mg/ml for all extracts.

4.3.2. Culture Preparation for Different Microbial Strains

Antimicrobial activity was evaluated against various bacterial strains obtained from commercial sources including *Escherichia coli* (*E. coli*), *staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Klebsella pneumonia* (*K. pneumonia*), *Acetobactrus baumannii*, *Morganella morganii*, and *Haemophilus influenza (H. influenza)* by using agar well diffusion method. Inocula of all microbes were prepared in sterilized Lauria-Bertini media gl^{-1} (SIGMA life science) in separate test tubes, which were then placed in a shaker incubator at for 24 hours containing approximately 10^8 cfu/mL.

4.3.3. Agar-Well Diffusion Assay for Antimicrobial Activity

Agar well diffusion technique was used for evaluating antimicrobial activity. Lauria-Bertini agar media was prepared and autoclaved at 121°C for 15 minutes which was then cooled and poured in Petri plates. The wells of 9 mm were bored in each plate and were inoculated with 75-µl inoculum and 100 µl extract and incubated at 37°C for 24 hours. After 24 hours zones of inhibition were measured and expressed in millimeters.

4.3.4. Antioxidant Activity

The ferric ion reducing power capability of the extract was determined using a modified method [40]. First, the sample was diluted 3-4 times with the same solvent in which it was extracted. Then five different concentrations were prepared by serial dilution while the volume of sample was kept constant to 750 µl. Each sample (750 µl) was mixed with the same quantity of phosphate buffer (0.2 M, pH 6.6) and 1% potassium ferricyanide (a source of ferric ions). The solution was then left into the incubator at 50°C for 20 minutes after which (750 µl) Trichloroacetic acid (10%) was added to stop the reaction and was then centrifuged at 3000 rpm for 10 minutes. The upper layer (1.5 mL) was separated and mixed with equal amount of distilled water and 0.1 ml $FeCl_3$ solution (0.1%). A blank was also prepared using different concentrations of ascorbic acid as standard using the same procedure and the absorbance was measured at 700 nm.

4.4. Statistical Analysis

The data were analyzed by one-way analysis of variance (ANOVA) using the statistical software package SPSS V.21.0 (SPSS, Chicago, IL, USA). Similarly, the mean separation was calculated using the Duncan multiple range test, and different letters were used for the statistically significant differences at $p > 0.05$.

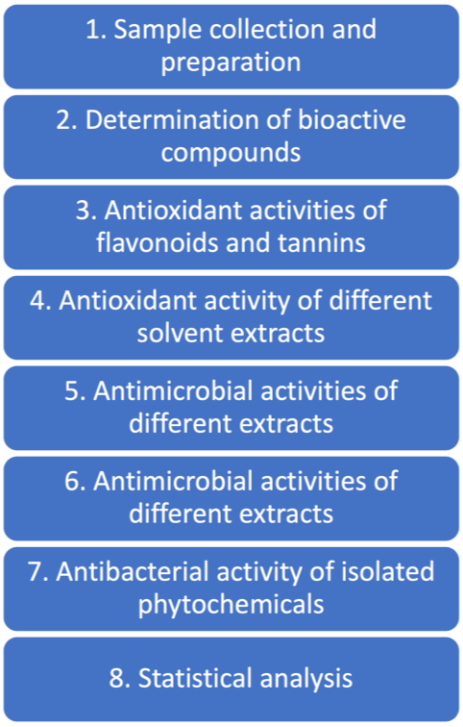

Fig. 6 Schematic representation of the study

5. Conclusions

From the current study it is concluded that an ethanol extract from the leaves of *T. hybridum* showed the highest antibacterial potential against the selected human pathogens as compared to those of the standards used. Gram-positive bacteria were found to be more susceptible to gram negative. The same extract was proven to be active in antioxidant assays. Interestingly, the leaves part of *T. hybridum* has more active phytochemicals such as tannins and flavonoids as compared to root and stem parts. The therapeutic potentials of *T. hybridum* include antimicrobial and antioxidant properties and these might be due to the presence of high concentrations of polyphenolic compounds like tannins and flavonoids and their derivatives. According to the Go-No-Go strategy for natural products, if the pure compound is less potent compared to crude extract, crude extract can be considered as a drug. The main limitation of this study is that Therefore, the present study encourages further investigation of the leave ethanol extracts of this valuable medicinal to help in combating several diseases, especially in the Asian developing countries.

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