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Phytochemical investigations and biological work on aerial parts and roots of *Trigonella polycerata*

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Abstract: The purpose of this study was to purify the phytoconstituents and to explore the antibacterial, antifungal, phytotoxic and cytotoxic potential of dichloromethane and methanol extracts of aerial and root parts of *Trigonella polycerata*. The phytochemical study on methanol extract of aerial parts of the plant led to the isolation and purification of seven compounds that were identified as 3,4-dimethoxycinnamaldehyde, Trigocoumarin, 6,7,8-trimethoxycoumarin, Penduletin, 5-hydroxy-3,6,7,4′-tetramethoxyflavone, 3,5,7-trihydroxy-6,4′-dimethoxyflavone and 5-hydroxy-4′,7-dimethoxyflavone. These structures were elucidated by interpretation of EI-MS and NMR spectral data. The plant aerial parts methanol extract (TPAM) demonstrated higher antibacterial (78.99%), phytotoxic (85% growth regulation at 1000µg/mL) and cytotoxic activities (LD_{50}: 45.643µg/mL). While the methanol root extract (TPRM) was highly active against bacteria's; *Salmonella typhi* (71.56%), *Staphylococcus aureus* (70.15%), *Escherichia coli* (69%), fungi like *Candida albicans* (70.21%) and moderately active against Brine shrimp larvae (LD_{50}: 125.663µg/mL). The dichloromethane aerial (TPAD) and root (TPRD) extracts exhibited significant antibacterial (78.03% and 50.21% inhibitions respectively) and phytotoxic (55% growth regulation at 1000µg/mL) potential. Only TPAD indicated the best inhibition against fungi; *Aspergillus flavus* (75.31%) and moderate inhibition against *Microsporum canis* (42.21%). This phytochemical and biological work is the first time reported in *Trigonella polycerata.*

Keywords: Aerial parts, antifungal activity, phytotoxic activity, *Trigonella polycerata*.

INTRODUCTION

Trigonella polycerata also known as *Trigonella monantha* grows annually in the flowering season from March to April in cultivated gram, wheat and rice paddies in Pakistan, India, and Afghanistan. This species is distinguished from ssp. *monantha* due to pedunculate inflorescence (Zandi *et al*. 2017). The plant, *Trigonella polycerata* has traditionally been used to cure various skin diseases. The seeds and whole plant of *Trigonella polycerata* are used as anti-hyperglycaemic, anti-hypercholesteraemic, and lactation stimulants (Zerabruk and Yirga 2012). The Mediterranean region is known to be the natural habitat of the genus *Trigonella*. The species of this genus are well reputed for their pungent, aromatic, highly nutritive, multi-therapeutic properties and serve industrial, culinary and medicinal purposes. Various pharmacological activities like antibacterial, antifungal, antioxidant, anticancer, anti-diabetic, analgesic and anti-inflammatory activities are reported in many plants of genus; Trigonella. Trigonella plants are marked with various groups of phytochemical constituents such as saponins, furostanol glycosides, sterols, triterpenoids, alkaloids and flavonoids. Lactation-stimulating oils and multiple gums are obtained mainly from seeds of these species (Bahmani *et al.* 2016).

The current study is designed to evaluate the phytochemical and biological potential of *Trigonella polycerata* based on folklore uses of Trigonella species.

MATERIALS AND METHODS

Chemicals & Apparatus

Chemicals such as methanol, chloroform, dichloromethane, and conc. sulphuric acid of Merck, Germany were used. The apparatus like Analytical balance (AX -200, SHIMADZU, Japan), Atomizer (CAMAG Switzerland) with 100ml flask (SCHOTT, Germany), TLC plates coated with silica gel 60 F_{254} (20×20 cm) of Merck, Germany, TLC tank (DESAGA, Germany), Oven (Memmert, U.K.), UV/VIS Spectrophotometer (Lambda 25, Perkin Elmer, U.S.A), glass columns (Quickfit, England), Mass spectrometer (JEOL JMS-600H, Japan), NMR (AVANCE AV-600

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CRYO PROBE, Bruker, U.S.A) and Rotavapor R-200 with recirculating chiller B-740 (Buchi, Switzerland) were used to carry out the research work.

Collection and identification of plant

The plant was collected in April 2015 from the surroundings of district Khanewal, Punjab (Pakistan), growing in gram and rice fields and authenticated as *Trigonella polycerata* by a Taxonomist; Prof. Dr. Zafrullah Zafar, Institute of pure and applied Biology of Bahauddin Zakariya University, Multan. The voucher number "Stewart 423" was allotted and deposited on herbarium.

Preparation of extracts

The aerial parts and roots of *Trigonella polycerata* were shade-dried separately for 15 days, then grinded to obtain a fine powder. The fine powder (500gm) of aerial parts and root was subjected to extraction separately by maceration with dichloromethane and methanol solvents successively. The extracts were concentrated under a vacuum at 35°C by Rotavapor. The extracts were collected and given the codes; TPAD (*T. polycerata* aerial parts dichloromethane extract), TPAM (*T. polycerata* aerial parts methanol extract), TPRD (*T. polycerata* root dichloromethane extract) and TPRM (*T. polycerata* root methanol extract).

Antibacterial activity

Antibacterial activity of aerial parts and root extracts of *Trigonella polycerata* was evaluated by using agar well diffusion assay. The bacterial strains; *Bacillus subtilis* (NCTC 8236), *Eschericha coli* (NCTC 10418), *Shigella sonnei, Staphylococcus aureus* (NCTC 6571), *Salmonella typhi* and *Pseudomonas aeruginosa* (ATCC 10145) were used as test microorganisms. The inoculum of bacterial culture containing 106cfu/ml was spread out over the nutrient agar plates. Six wells of 8 mm were bored in the agar medium with 6mm sterile cork borer. The wells were filled with plant extracts of 100µl (0.1mg/ml) and then allowed to diffuse for two hours at room temperature. The well plates were placed for incubation for 24 hours at 37°C temperature. The ampicillin and ciprofloxacin were considered as standard drugs. The %inhibition showed by plant extracts and standard drugs were calculated after incubation. The procedure was repeated three times and data was expressed in mean ± standard deviation (Gummuluri *et al.* 2019).

Antifungal activity

Antifungal activity of aerial parts and root extracts of *Trigonella polycerata* was also evaluated by using agar well diffusion assay. The fungal strains like *Candida albicans* (ATCC 90028)*, Aspergillus flavus* (PTCC 5018) and *Microsporum canis* (PTCC 5069) were used as test microorganisms. The Amphotericin- B, clotrimazole and miconazole were considered as standard drugs. The rest of the procedure is the same as discussed in antibacterial activity.

Phytotoxic activity

The phytotoxicity was evaluated by *Lemna minor* bioassay. The E-medium for the assay was prepared with its pH range 5.5-6.0 after addition of potassium hydroxide pellets. The eight sets of 20 vials each for 500, 50, 5ppm and standard control were prepared. The various graded extracts concentrations (1000, 100 and 10µL) were put in 500, 50 and 5ppm vials and placed overnight to evaporate. Each vial was filled with E-medium (2mL) and a rosette of 3 fronds of a *Lemna minor*, put the vials in 2cm water-filled glass dish, and sealed by stopcock. Then the glass dish was placed in a growth chamber for seven days under fluorescence and incandescent light at 26°C. The no. of fronds were counted and recorded on 3rd & 7th day (Younus *et al.* 2021).

Brine-shrimp lethality assay

The artificial seawater was prepared by adding 3.8g of NaCl in one litre H_2O, filtered and then eggs of shrimps were added. After hatching, larvae were matured within two days at 22-29° temperature. The vials were prepared at 10, 100, 1000µl/mL concentrations for test. Each fraction was made in triplicate and 20mg plant extract was added in 2mL solvent. The sample was transferred in 5, 50, 500µL concentrations to vials corresponding to 10, 100, 1000µl/mL respectively. Etoposide was taken as a standard drug. In each vial, 10 shrimps and 5mL sea water (30 shrimps/dilution) were added and taken under illuminating light for 24h. The survived shrimps were numbered and used for Probit analysis (Finney Computer program) (Yadav and Mohite. 2020).

Isolation of Compounds

Based on TLC analysis, 25gm of TPAM was fractionated by open column chromatography with the stationary phase; silica gel 60 (Size: 63-200µm) and mobile phase; $CHCl_3$: MeOH: Water with the ratios; 80:20:02➜ 75:25:03➜ 70:30:04➜ 65:35:05➜ Pure MeOH via stepwise elution. Seven fractions TPAM-1 to TPAM-7 were obtained. The fraction; TPAM-2 (4.34 g) was further resolved into four sub fractions; TPAM-2A to TPAM-2D. The fractionation of TPAM-2B (756 mg) was resulted in TPAM-2B1 to TPAM-2B5. The fraction; TPAM-2B2 (315 mg) was further chromatographed using silica gel 60 (Size: 40-63µm) and mobile phase; $CHCl_3$: IPA (90:10) that afforded the pure compound-1 (10 mg). When the fraction; TPAM-2D was dissolved in MeOH then, compound-2 (11 mg) was settled down. The upper MeOH soluble portion was separated carefully, and the compound was washed by adding a sufficient volume of MeOH. The TPAM-4 (4.33gm) resolved into TPAM-4A to TPAM-4D using open column chromatography. The TPAM-4B (856 mg) was further fractionated into TPAM-4B1 to TPAM-4B3. The TPAM-4B1 (134 mg) was

subjected to gel filtration chromatography that yielded compound-3 (16 mg) and 4 (13mg). TPAM-4B3 (389 mg) was chromatographed using silica gel 60 (40-63 µm) and a solvent system; $CHCl_3$: MeOH with varying ratios; 80:20➔70:30 that gave a pure compound-5 (16 mg) and two fractions TPAM-4B3a and TPAM-4B3b. The fractionation of TPAM-4B3b (205 mg) on silica gel 60 (40-63 µm) and mobile phase; $CHCl_3$: MeOH (70:30➔60:40) yielded a compound-6 (14 mg). On drying the fraction; TPAM-7 (5.125 g), yellow crystals of Compound-**7** (9 mg) were obtained that were separated and cleaned between the folds of filter paper.

Spectroscopic data of isolated compounds

Compound-4: Pale yellow solid; Yield: 13mg; HR-EI-MS: *m/z* 344.0861; UV (CH_3OH, λ_{max}, nm: 358 (3.22) and 273 (2.72); IR (KBr, $v_{max,}$ cm^{-1}): 3331 (OH), 1649 (C=O), 1508 (Ar), 1368 and 893; Sharaf *et al.* 1992 mentioned the spectral data that is similar with ^{1}H NMR and ^{13}C-NMR spectra of Compound-4 .

Compound-5: White yellowish solid; Yield: 16mg; HR-EI-MS: *m/z* 358.1059; UV (CH_3OH, λ_{max}, nm: 361 (3.78) and 269 (3.99); IR (KBr, $v_{max,}$ cm^{-1}): 3509 (OH), 1728 (C=O), 1598 (Ar), 1362 and 871. The rest of spectrum is identical with previous mentioned reports (Çitoglu *et al.* 2005).

Compound-6: Amorphous light yellow powder; Yield: 14 mg; El-MS *m/z* $[M]^+$ 330 (100), 315, 318, 183, 135; HR-EI-MS *m/z* 330.0753; NMR data is same with previous reports on Compound-6 in the solvent; DMSO (Horie *et al.* 1997). But the data for $CDCl_3$ solvent is given as; ^{1}H NMR: (400 MHz, $CDCl_3$) δ (ppm) 8.09 (2H, d, J= 8.7 Hz, H-2'/6'), 6.88 (2H, d, J = 8.7 Hz, H-3'/5'), 6.71 (IH, s, H-8), 3.71, 3.89 (3H each, s, OMe-4′, 6) along with -OH signals; ^{13}C-NMR: (100 MHz, $CDCl_3$) δ (ppm) 56.8 and 55.4 (MeO-6 & MeO-4′), 158.1 (C-2), 152.5 (C-7), 116.4 (C-3′/5′), 132.1 (C-2 / 6′), 122.8 (C-l′), 158.5 (C-4′), 149.3 (C-9), 131.5 (C-6), 176.9 (C-4), 104.1 (C-10), 153.5 (C-5), 137.5 (C-3), 92.8 (C-8). The compound-1, 2, 3 and 7 were elucidated based on UV, IR, NMR, MS and Co-TLC analysis.

STATISTICAL ANALYSIS

The data was presented in mean ± Standard deviation (SEM). The LD_{50} values were obtained from the best-fit line by regression analysis. One-Way ANOVA was carried out from Graph Pad Instat R version 2.05 (UK) to test the significant values. $P<0.05$ was regarded as a significant.

RESULTS

Identification of compounds 1-7

Interpretation of the corresponding spectral data and its comparison with previously reported information supported the identification of the purified compounds as 3,4-Dimethoxycinnamaldehyde (1), 3-(ethoxycarbonyl)-4-methyl-5,8-dimethoxy coumarin (Trigocoumarin) (2), 6,7,8-trimethoxycoumarin (Dimethylfraxetin) (3), 5, 4′ -dihydroxy-3, 6, 7-trimethoxyflavone (Penduletin) (4), 5-hydroxy-3,6,7,4′-tetramethoxyflavone (5), 3,5,7-trihydroxy-6,4′-dimethoxyflavone (6) and 5-hydroxy-4′,7-dimethoxyflavone (7).

Antibacterial Activity

TPAM and TPRM (methanol extracts) exhibited significant antibacterial activities with 78.99% and 69% inhibitions against *Escherichia coli,* 74.20% and 71.56% against *Salmonella typhi*, 78.99% and 35.74% against *Shigella sonnei,* 58.99% and 70.15% inhibitions against *Staphylococcus aureus* respectively. Only TPRM showed 59% inhibitory activity against *Pseudomonas aeruginosa*. Both extracts were found to be inactive against *Bacillus subtilis.* The dichloromethane extracts; TPAD and TPRD showed 45.81% and 37.74% inhibitions against *Salmonella typhi,* 59.21% and 34.56% against *Shigella sonnei,* 78.03% and 50.21% inhibitions against *Bacillus subtilis* respectively. TPRD was inactive against *Pseudomonas aeruginosa, Escherichia coli* and *Staphylococcus aureus* while TPAD indicated no potential against *Pseudomonas aeruginosa.* The results of antibacterial activity are presented in table 1.

Antifungal activity

TPRM was found to be highly active with 70.21% inhibition while other extracts showed lower potential against *Candida albicans.* Against *Aspergillus flavus* and *Microsporum canis,* TPAD showed maximum antifungal activity with 75.31% and 42.21% inhibitions respectively. The remaining extracts exhibited lower or no activity against *Aspergillus flavus* and *Microsporum canis.* The results of antifungal activity are expressed in table 2.

Phytotoxic activity

The methanol plant extracts; TPAM and TPRM exhibited significant phytotoxic activity with 55% growth regulation. While dichloromethane extracts; TPAD and TPRD identified less activity at 100µg/mL concentration. The extract; TPAM indicated excellent phytotoxic potential with 85% growth regulation and other extracts; TPAD, TPRM and TPRD showed maximum 55% growth regulation at 1000µg/mL concentration after comparison with the standard drug; paraquat. All the extracts were less active at a dose of 10µg/mL. The results of phytotoxic activity are expressed in table.3.

Brine-Shrimp Lethality bioassay

The methanol plant extracts; TPAM indicated highest cytotoxic potential against brine shrimp larvae at all doses; 10, 100, 1000µg/mL with LD_{50}: 45.643µg/mL. While TPRM responded moderate cytotoxic potential with LD_{50}: 125.663µg/mL. The dichloromethane extracts;

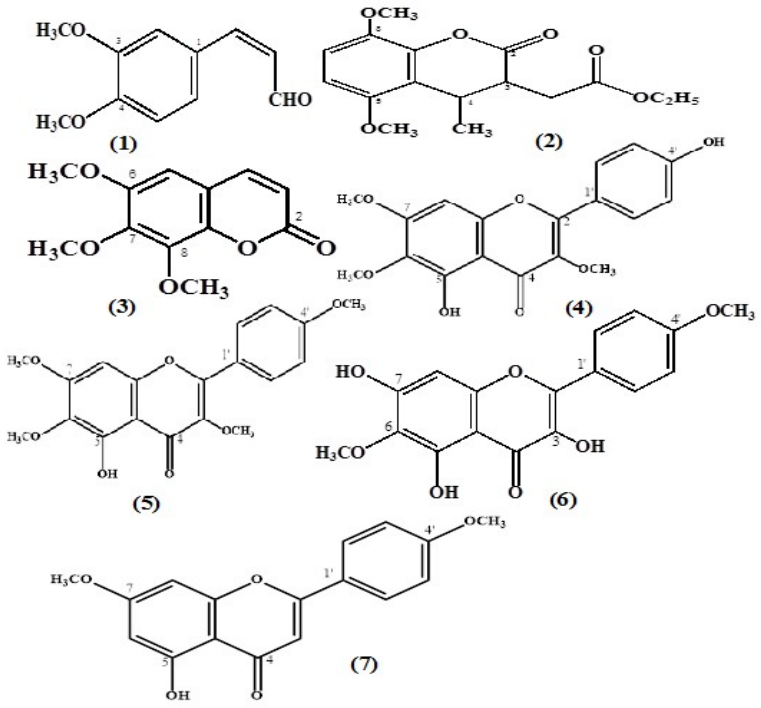

Fig. 1: Structures of the purified compounds (1-7)

TPAD and TPRD showed lower activity with LD_{50} values of 430.675 and 630.435µg/mL respectively. The results of brine-shrimp lethality bioassay are presented in table 4.

DISCUSSION

The consciousness level concerning the favoured prophylactic and therapeutic use of medicinal plants is rapidly rising due to the wide array of phytochemicals responsible for diverse biological activities. These phytochemicals are helpful to treat various communicable diseases with no or fewer side effects (Mayyas *et al.* 2021). The phytochemical research work on *Trigonella polycerata* led to the purification of one cinnamaldehyde derivative, two coumarins and four flavonoids.

The isolated compounds were already identified in various species of Fabaceae family like Trigocoumarin from *Trigonella foenumgraecum (*Nifras *et al.* 2021), 3, 4-dimethoxycinnamaldehyde from *Platymiscium gracile (*Cuellar *et al.* 2020), 6, 7, 8-trimethoxycoumarin from *Platymiscium floribundum* (Veloso *et al.* 2012) and 5-hydroxy-4′, 7-dimethoxyflavone from *Mimosa caesalpiniifolia* (Santos *et al.* 2015). But the flavonoids like Penduletin, 5-hydroxy-3, 6, 7, 4′-tetramethoxyflavone, 3, 5, 7-trihydroxy-6, 4′-dimethoxyflavone and four compounds mentioned above are first time reported in the plant; *Trigonella polycerata* of family Fabaceae.

Over the last 100 years, antibiotics have been essential for treating microbial infections and have extended life expectancy (Owusu *et al.* 2021). However, several microorganisms have developed resistance to the overuse and misuse of antibiotics among the general population. Hence, the efforts are being made to combat epidemic antibiotic resistance and to explore alternative sources of antimicrobial agents, such as medicinal plants (Guevara-Salazar *et al.* 2021). The results of antibacterial and antifungal activities of *T. polycerata* are consistent with many Trigonella species like *T. foenum-graecum, T. coerulescens, T. stellate* and *T. suavissima* that were found to be broadly active against various bacterial strains like *P. syringae, B. subtilis, E. coli, S. typhi* and *S. aureus.* In contrast, methanol extracts of some Trigonella plants demonstrated antifungal potential against *A. niger* and *F. solani* (Dangi *et al.* 2016). Moreover, isolated compounds like 3, 4-Dimethoxycinnamaldehyde, coumarins and flavonoids have pronounced antimicrobial potential that is mentioned in the previous reports (Guzman, 2014).

Cancer is a leading cause of death worldwide (Ferlay *et al*. 2021). Many approaches have been developed to reduce the threat caused by cancer. According to Stone *et al.* 2022, it's estimated that 60% of the new chemicals introduced between 1981 and 2002 were natural products or derived from natural lead compounds. The brine shrimp lethality bioassay is the most reliable, simple and inexpensive cytotoxicity screening process that is correlated with cytotoxicity and antitumor properties (Kapali and Sharma 2021).

Table 1: Results of antibacterial activity of *Trigonella polycerata* extratcs.

Extracts/ Standard	Antibacterial activity (% Inhibition at concentration of 0.1mg/mL)					
	Escherichia coli	*Salmonella typhi*	*Pseudomonas aeruginosa*	*Shigella sonnei*	*Bacillus subtilis*	*Staphylococcus aureus*
TPAD	49.03	45.81	-	59.21	78.03	66.21
TPRD	-	37.74	-	34.56	50.21	-
TPAM	78.99	74.20	-	78.03	-	58.99
TPRM	69	71.56	59	35.74	-	70.15
Ampicillin	93.65	87.32	91.41	92.76	94.11	91.41
Ciprofloxacin	93.66	91.34	90.02	96.33	93.73	95.23

Table 2: Results of antifungal activity of *Trigonella polycerata* extratcs.

Extracts/Standard	Antifungal activity (% Inhibition at concentration of 0.1mg/mL)		
	Candida albicans	*Aspergillus flavus*	*Microsporum canis*
TPAD	19.19	75.31	42.21
TPRD	33.19	37.19	-
TPAM	21.26	12.34	9.13
TPRM	70.21	23.66	22.31
Clotrimazole	95.11	-	-
Amphotericin-B	-	94.31	-
Miconazole	-	-	93.41

n = 3, TPAD: *T. polycerata* aerial parts dichloromethane extract, TPAM: *T. polycerata* aerial parts methanol extract, TPRD: *T. polycerata* root dichloromethane extract, TPRM: *T. polycerata* root methanol extract

Table 3: Results of Lemna minor phytotoxic activity of *Trigonella polycerata* extracts.

Extracts/Standard	Concentration (µg/mL)	No. of Fronds		% Growth Regulation
		Taken	Survived	
TPAM	10	20	16	20
	100	20	9	55
	1000	20	3	85
TPAD	10	20	18	10
	100	20	15	25
	1000	20	9	55
TPRM	10	20	17	15
	100	20	9	55
	1000	20	9	55
TPRD	10	20	19	5
	100	20	16	20
	1000	20	10	55
Control	-	20	20	0
Paraquat	0.015	20	0	100

Table 4: Results of Brine-Shrimp Lethality bioassay of *Trigonella polycerata* extracts.

Extracts/Standard	Concentration (µg/mL)	No. of Shrimps	No. of Survivors	LD_{50} (µg/mL)
TPAM	10	30	02	45.643
	100	30	0	
	1000	30	0	
TPAD	10	30	24	430.675
	100	30	22	
	1000	30	20	
TPRM	10	30	14	125.663
	100	30	11	
	1000	30	09	
TPRD	10	30	28	630.435
	100	30	24	
	1000	30	21	
Etoposide	7.4626			

This cytotoxicity assay was conducted on the basis of traditional significance and previous cytotoxicity analysis of genus *Trigonella*. The species of this genus are well reputed for significant cytotoxic effects. Diosgenin, a crystalline steroidal sapogenin isolated from *T. foenum-graecum* and *T. corniculata* causes the suppression of proliferation and has ability to stop invasion (Meghwal and Goswami 2012). Both methanolic extracts of *T. polycerata* showed significant cytotoxic activity. This work will surely provide a baseline for further anticancer research i.e. cell line assays.

Synthetic herbicidal agents are primarily used to control weeds but their overuse has increased the threats of environmental pollution and resistance against herbicides. Alternative cost-effective and eco-friendly weed control strategies are prime requirements worldwide. The phytotoxic plants contain such growth inhibitors in order to resolve the problem caused by synthetic herbicidal agents (Islam and Kato-Noguchi 2014). The current study on plant extracts indicated higher phytotoxic potential in *T. polycerata*. Further research is required to isolate and characterize the bio-herbicides in this plant.

CONCLUSION

The plant; *Trigonella polycerata* indicated promising antibacterial, antifungal, cytotoxic and phytotoxic potential that is assumed to occur due to the purified phyto-constituents. It will surely provide a base for further research to get the valuable antibacterial, antifungal, cytotoxic and phytotoxic agents.

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