Verbascum Thapsus (Mullein) Versatile Polarity Extracts: GC-MS Analysis, Phytochemical Profiling, Anti-bacterial Potential and Anti-oxidant Activity

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ABSTRACT
Verbascum thapsus is naturally grown in the Himalayas and widely used in herbal teas and traditional herbal medicine for its anticarcinogenic and anti-inflammatory properties. The present study was designed to majorly leaf extracts from Verbascum thapsus. All extracts were analysed for phytochemical properties, antioxidant capacity and antimicrobial potential against both Gram-positive and Gram-negative bacteria. Biochemical investigations and GC-MS analysis was used for identifying phytochemicals. DPPH assay, Kirby’s Disc Diffusion method (KDM), 96 well test, and Resazurin test were performed for antioxidant and antimicrobial investigation. Results indicate that verbascum thapsus grown in Pakistan is rich in alkaloids and phenols. Noteworthy antibacterial activity was observed against S. sonnei, L. lactis, B. subtilis, C. freundii, K. oxytoca, L. monocytogenes, and S. enterica. GCMS analyses of V. thapsus extracts revealed the presence of medically important bio compounds including Hexadecanoic acid, methyl es and Stigmasterol (antibacterial activity), 2(5H)-Furanone (appetite suppressant), 3-Hydroxy-beta-damascone (anti-inflammatory properties), Squalene (antiaging, anti-inflammatory, anti-acne, eczema), Vitamin E and 2-Methoxy-4-vinylphenol (antioxidants). Antioxidant radical scavenging activity was determined from acetone extract of V. thapsus. This study concludes remarkable bacterica and antioxidant potential in Verbascum Thapsus leaf extracts.

Key words: Verbascum Thapsus; GC/GC-MS; plant biotechnology, Microbiology.

INTRODUCTION
Cure of microbial ailments via plant extracts is in practice for centuries1 deals with infectious diseases because of their antibiotic and antioxidant potential. Verbascum thapsus also known as common mullein is utilized as traditional medicine and tea rich in antioxidants and antibacterial plants for long time1,2. More than one hundred accepted names are given to large genus Verbascum belonging from Scrophulariaceae family in plant databases3. Leaves and flowers of V. Thapsus have expectorant and anti-inflammatory potential in herbal medicines4. V. thapsus reduces the severity of respiratory conditions including bronchitis and asthma along with reported effectiveness against haemorrhoids, fungal infections, and diarrheaa5. Yet, the detailed phytochemical spectrum in versatile solvents remains relatively understudied. Plants from Verbascum genus have exhibited inhibitory activity against murine lymphocytic leukaemia, several strains of influenza viruses, Trichomonas vaginalis, etc.6. Plants of Verbascum species also have potential anticarcinogenic properties6. Verbascum Thapsus has been used as herbal treatment of several disorders, but no FDA approved drug is yet available. Prominent medicinal uses include treatment for Parkinson’s disease, diabetes, bronchitis, asthma, joints and stomach pain, and skin issues etc7. Flower extracts of V. Thapsus also show promising concentrations of Terpenoids. Iridoid glycosides, Lignan glycosides, phenylethanoid glycosides, sterones and saponins etc8. Verbascum thapsus is extensively grown in temperate areas of Pakistan and its medicinal importance prompted us to study it comprehensively. The phytochemistry of Verbascum thapsus is usually studied in a single solvent i.e., methanol, in the past decade8 and not a single study discusses it in all five range of solvents. Therefore, in continuation of prior studies, the present study aims to explore multiple aspects i.e., phytochemical analyses of Verbascum thapsus and quantitative/qualitative investigations via GCMS and its antibacterial activities in five different solvents (Figure 1).

RESULTS AND DISCUSSION
Verbascum thapsus is used in the treatment of pulmonary problems, inflammatory diseases, asthma, spasmodic coughs, diarrhoea, tuberculosis, and migraine headaches, it also possesses antiviral, anti-cancer, and antibacterial potential9,10. Numerous bioactive agents have been extracted from oils and other extracts of Verbascum species11.

Phytochemical analysis
The phytochemical profiling of Verbascum thapsus extracts in methanol, ethanol, water, acetone, and hexane was performed to reveal phenol, alkaloids, tannins, saponins, cardiac glycosides, flavonoids, water-soluble phenols, water-insoluble phenols, triterpenoids, free anthraquinones, and combined anthraquinones (Table 1). Alkaloids and phenols were present in most extracts while saponins and combined anthraquinones were only detected in water-based solvents. The highest flavonoid content was present in acetone extract (3.1969 ug/ml) and the highest phenol content (2.770 ug/ml) was present in methanol extract (Figure 2).
Figure 1: Layout of phytochemical, antioxidant and antibacterial analyses of Verbascum Thapsus.

Figure 2: Total flavonoid and phenol estimation in Methanol, ethanol, water, acetone and hexane extracts of Verbascum Thapsus. X axis indicate the extracts in different solvents whereas concentration of flavonoid and phenol is represented in ug/ml on y axis.
Phenolic and flavonoid compounds enhance the antioxidant activity of plants and are pharmaceutically important. Enormous antimicrobial, antioxidant, antitumorigenic, cardiovascular protective, immune-boosting, and anti-inflammatory effects are highlighted in earlier studies. Sufficient phenol and flavonoid percentage may contribute to prior mentioned pharmaceutical benefits. Several Verbascum species like V. phlomoides, V. pestalozzae, V. detersile, V. densiflorum contain important phytochemicals in the phenol range that makes them pharmacologically important.

**GCMS analysis**

Methanolic extract of Verbascum thapsus showed the presence of 25 phytochemicals, while 41 phytochemicals were detected in ethanolic extracts. In the water extract, 15 phytochemicals were detected while 29 phytochemicals were detected in acetone extracts (Figure 3, Supplementary Table 2). Fifteen phytochemicals were detected in hexane extract. Mass spectrometric analysis of V. thapsus extracts revealed the presence of many important compounds. Ethanolic extract, alone, contains 41 bioactive compounds. However, the least number of metabolites were present in the water and hexane-based extract of V. thapsus. Some prominent compounds include 2-Methoxy-4-vinylphenol (antioxidant, flavouring agent, anti-inflammatory effect), Phosphonic acid (medical imaging, pro-drug), Stigmasterol and Hexa-decanoic acid, methyl est (antibacterial activity), n-Hexa-decanoic acid (cosmetics) and Vanillic acid (flavouring agent), 2(5H)-Furanone (appetite suppressant), 3-Hydroxy-β-damascone (anti-inflammatory properties), Mequinol (skin depigmentation), Fluoroacetic acid, dodecyl ester (pesticide), Squalene (anti-aging, anti-inflammatory, acne, eczema), Vitamin E (antioxidant), Hydroquinone (hyperpigmentation) and Phytol (fragrance agent, transcription modulator). We would like to report many chemicals that did not match any reference molecule in GCMS library. These may have potential antioxidant, antimicrobial or any other activities. Therefore, it is of vital importance to identify and separate the unmatched compounds (Supplementary Table 2) via further analytical techniques like Mass spectrometry and flow cytometry. Further experiments could be conducted to evaluate the pharmacological potential of these unknown compounds present in Verbascum Thapsus extracts.

Plants use metabolites for their defence against pathogenic microbes using various strategies. These metabolites contribute to the intervention of microbe invasion and repel herbivores from potential harm they may pose to plant survival and activity. New antibiotics are demanded worldwide to answer the question arise by antibiotic resistant variants. Pure plants and herbal extracts are used universally to design chemicals of pharmaceutical importance.
Antibacterial activity

Antibacterial experiments (well plate method, Kirby’s disc diffusion method, and resazurin absorption method) were performed on ten bacterial strains. Gram-positive strains included: Bacillus subtilis (ATCC_6051), Micrococcus luteus (ATCC_4698), Staphylococcus aureus (ATCC_25923), Lactococcus lactis (ATCC_LMO230), Listeria monocytogenes (ATCC_LM21) while the gram-negative bacterial strains included: Shigella sonnei (ATCC_25931), Salmonella enterica (ATCC_14028), Escherichia coli (ATCC_25922) and Klebsiella oxytoca (ATCC_43863). Overall maximum antimicrobial activity was displayed by ethanol and methanol-based extracts of V. Thapsus against most of the bacterial strains. Maximum percent growth inhibition of S. sonnei was produced by ethanol extract at 1000ug concentration. However, both ethanol and methanol extracts at 1000ug and 500ug concentration showed growth inhibition of L. lactis and C. freundii. Acetone based extract inhibited the growth of L. monocytogenes, L. lactis, and B. subtilis. On the other hand, water-based extracts of V. thapsus showed the least bacterial growth inhibition against all the bacterial strains.

**Kirby disk diffusion method** was used to study the bacterial growth inhibition by plant extract in various solvents. Ethanol, methanol, and acetone-based extracts showed significant antimicrobial activity against most of the bacterial strains while water and hexane-based extracts showed least antibacterial activity as compared to control treatment. Maximum inhibition was observed against C. freundii, M. luteus, S. sonnei, and K. oxytoca (Supplementary Table 3). The average diameter (mm) of inhibition zone in culture plates was recorded in triplicate and the mean value ± standard deviation of diameter has been presented in Supplementary files as table 3. Verbascum thapsus total extract showed amazing MIC in terms of percentage growth inhibition against L. lactis, E. coli, and K. oxytoca. (Figure 4). More than seventy percent of bacterial growth of L. lactis was inhibited via V. thapsus extract at 100ul concentration.

**Well plate method and Kirby’s disc diffusion method** indicated maximum antimicrobial activity of ethanol, methanol, and acetone extracts of V. thapsus (Figure 5). Our results support previous findings18,20,21 where ethanolic and methanolic extracts performed better in antibacterial action as compared to aqueous extracts. One way ANOVA results indicate a significant difference (p-value: <0.05) in the antibacterial activity of Verbascum extracts (Figure 6).

Another experiment of DMSO mediated solubilization was conducted. Studies report prominent antimicrobial action of V. thapsus extracts against S. epidermidis, S. aureus, K. pneumonia, and E coli strains18. The present study explored the promising antibacterial activity of V. thapsus against L. lactis, E. coli, and K. oxytoca. Our results not only validate the research findings of the antibacterial properties of V. Thapsus22 but also coincide with the other Verbascum species i.e., Verbascum macrurum21. It is therefore indicated that significant antimicrobial potential in Verbascum species ranks them high as medicinal plants19,24.

**DPPH antioxidant activity**

The use of DPPH to evaluate antioxidant activity in plant extracts. The highest antioxidant activity was recorded in acetone extract of V. thapsus (651.03125umol/L) followed by water-based extracts (Figure 7). The antioxidant order of V. thapsus extract was acetone > water > hexane > ethanol > methanol. The lowest IC50 value shows the highest antioxidant potential of extracts. This indicates the presence of free radical scavenging active metabolites and makes this plant a good antioxidant25,26. On the contrary, prior studies indicated the maximum antioxidant potential of Verbascum plant species in water and methanol extracts27,28. For long, Verbascum species have been used in several regions across the globe for their antitumorigenic, anti-inflammatory, and anti-spasmodic effects along with diminishing migraine symptoms and wound healing12,23. Several other investigations also highlighted the antioxidant activity of Verbascum species in hydrophilic solvents26,29. Therefore, the antioxidant activity of V. thapsus revealed from our study is supported by other studies. The presence of phenolic compounds and antioxidants may provide advantageous health and medicinal benefits by Verbascum consumption in either raw or processed form after intensive tests and trials30.
METHODS

Plant material

Verbascum thapsus plants were collected during July 2016 from district swat (35.2227° N, 72.4258° E), a district in Khyber Pakhtunkhwa (KPK) province Pakistan, and identified by the national herbarium later brought to the laboratory (Antimicrobial Biological Laboratory; AMBL, International Islamic University Islamabad, Pakistan).

Extract preparation and Filtration

Shade dried Verbascum thapsus plants were chopped and ground to a fine powder. The prepared powder (200g) was passed through a sieve (pore size 20-25um) followed by dissolving in five solvents (each solvent 1000 mL) of different polarities including Methanol, Ethanol, Water, Acetone, and Hexane obtained from Stockbridge Medicinal and aromatic Lab, University of Massachusetts Amherst, USA. Plant powder was separately macerated in each of the solvents using a rotary evaporator and shaking at room temperature for two days i.e., 48 hours and then filtered through a Whatmann No. 41 paper.

Phytochemical analysis

Preliminary qualitative analysis

The plant extracts prepared in different solvents were subjected to preliminary qualitative analysis by adopting well-established procedures7. All the phytochemical tests were performed to confirm the presence of saponin, phenolic compounds, water-soluble phenol, water-insoluble phenol, flavonoids, polysteroids, terpenoids, cardiac...
glycosides, free anthraquinones, combined anthraquinones, tannins, and alkaloids in the tested plant samples.

Quantitative analysis

**Phenols**

Phenols were quantified by following the standard procedures. Briefly, 75 µL deionized distilled water (ddH2O) was added to each well of 96-well plate. 25 µL Folin C (F–C reagent, Sigma-Aldrich) was also added to each well of a plate (diluted 1:1 v/v with ddH2O) and left it to stand for 6 min. 100 µL of Na2CO3 (75 g/L) was added to each well. After thorough mixing, plates were put in dark for 90 min. Each of the samples was repeated in triplicate. A spectrophotometric microplate reader (SPECTRA MAX M2e) was used to take readings at 765 nm. Gallic acid was used as a standard at 12.5–400 µg/mL and a standard calibration curve were generated. Phenols were determined as µg of Gallic acid equivalents / mL, which was calculated via formula, 

\[ y = 0.5377x + 0.316 \]

where y is the absorbance at 510 nm and x is the amount of gallic acid equivalent in µg/mL². 

**Flavonoids**

Flavonoids were quantified by following the standard procedures. 100 µL ddH2O was dispensed in each of the 96 wells of the well plate. 10 µL of NaNO2 (50 g/L) and 50 µL of AlCl3 (100 g/L) were added to the mixture and left for 6 minutes. Later, 50 µL of NaOH (1 mol/L) and 50 µL of ddH2O were added to each well, and the plate was shaken for 30 sec, and absorbance was measured at 510 nm using SPECTRA MAX M2e plate reader. Catechin was used as a standard at 5–500 µg/mL to generate calibration curve and flavonoids content of plant extract was expressed in µg of Catechin equivalents / mL and were calculated by the formula, 

\[ y = 0.6053x – 0.0567 \]

where y is the absorbance at 765 nm and x is the amount of gallic acid equivalent in µg/mL².

**DPPH Antioxidant assay**

DPPH radical scavenging assay by Bersuder, Hole and Smith 1998 was used to determine the antioxidant activity of plant extract. 25uLof Plant extracts were left to react with free DPPH (200 µL) prepared in ethanol for 6 hrs in dark in all 96 wells. Ascorbic acid was used as a standard at 50–1000 µmol/L concentrations to generate a calibration curve. It was dissolved with DMSO alone to act as a negative control for percentage calculation of plant extract radical scavenging activity. Absorbance was measured at 517 nm².

**Gas Chromatography-Mass Spectrometry (GC/MS) Analysis**

The phytochemical investigation of plant crude extract was performed using GC-MS equipment (Bruker Scion 456 GC, EVOQ triple quadrupole GC/MS/MS) following the standard procedures. The experimental conditions of the GC-MS system were as follows: Column: 15m, 0.25mm inner diameter, 0.25mm film thickness. The flow rate of the mobile phase (carrier gas: He) was set at 1.5 mL/min. In the gas chromatography part, temperature-programmed (oven temperature) was 45°C hold 3 min-raised to 250°C at 8°C/min hold 10 min and injection volume of 1 ul using the varying split ratio (5:1/15:1/20:1). Samples dissolved in all five solvents were run fully at a range of 45–350 m/z and the results were compared by using Software MSWS 8, Automated Mass Spectral Deconvolution and Identification System (AMDIS) for GC-MS and NIST library.

**Antibacterial activity**

**Bacterial cultures collection and Inoculum preparation**

Antibacterial experiments were performed on ten bacterial strains. Gram-positive strains included: *Bacillus subtilis* (ATCC_6051), *Micrococcus luteus* (ATCC_4698), *Staphylococcus aureus* (ATCC_25923), *Lactococcus lactis* (ATCC_LMO230), *Listeria monocytogenes* (ATCC_LM21) while the gram-negative bacterial strains included: *Shigella sonnei* (ATCC_25931), *Salmonella enterica* (ATCC_14028), *Escherichia coli* (ATCC_25922) and *Klebsiella oxytoca* (ATCC_43863).

All these bacteria were separately grown on Tryptic Soy Broth (TSB) medium (Thermo Fisher Scientific, USA) and incubated at 26 ± 2 °C for twenty-four hours. All the pure bacterial cultures were stored at -4 °C till further use in experimentation. Bacterial inoculum was prepared by suspending the pure bacterial colonies in 25 mL capacity lid vials containing sterilized nutrient broth medium followed by incubation at 26 ± 2 °C on a rotary shaker for 24 hours. The concentration of each of the bacterial inoculum was maintained at 10⁷ to 10⁸ CFU/mL.

**Determination of Minimum Inhibitory Concentrations (MIC) and percentage inhibition / antibacterial activity**

The MIC of *Verbascum thapsus* was investigated by following three methodologies including 96 well test, Kirby-Buyer disk diffusion and resazurin-based well plate microdilution method.
96 well sterile microtiter tray assay
96 well sterile microtiter tray assay was reformed by adopting the standard operating procedure\(^1\). 100 µL TSB medium, 100 µL of plant extract fractions at 5 dilution levels (1000 µg, 500 µg, 250 µg, 125 µg, and 62.5 µg) and 50 µL of each bacterial culture (105 to 106 CFU/mL concentration) were loaded in the wells\(^2\). A well-containing TSB medium only (lacks Plant extract and culture) was maintained as a double negative well to check the sterility of the medium. Another well-contained TSB medium and bacterial inoculation (lacks Plant extract) to check average bacterial growth trends as single negative control wells. Plates were covered, sealed, and incubated at room temperature for 24 hours. Standard readings for MIC were taken by recording the liquid medium and total plant extract was added to the wells\(^3\) 24 hours. The resazurin-based Well Plate Microdilution test was performed using the SPECTRA MAX M2e plate reader 36. Absorption readings of resazurin developed color intensity was observed against most bacterial strains and has the potential for development as a bactericide and medicinally important drug.

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ADDITIONAL INFORMATION
Conflicts of interest/Competing interests: Authors declare no conflict of interests.

Availability of data and material: GCMS Data has been given in supplementary files further materials or relevant data will be available upon request to author.

Code availability: Not Applicable.

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GRAPHICAL ABSTRACT

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