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Chemo profiling and In-vitro Antioxidant, Anti-inflammatory Activities of Vilwadi Tablet-An Important Ayurvedic Formulation

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Abstract
Ayurvedic formulations have attracted great interest due to their potential biological activities and lesser side effects. Vilwadi tablet is an important antitoxic polyherbal ayurvedic formulation which is widely used in managing various bite injuries and systemic disorders, helps in the treatment of toxic symptoms from infections, improve appetite and overall digestive function. However, this formulation is not much scientifically validated and the chemical constituents responsible for their biological activities are not fully known. The objective of the present study is to identify the unique bioactive compounds present in Vilwadi tablet. Preliminary phytochemical screening showed that the formulation contains important secondary metabolites such as alkaloids, flavonoids, phenolic compounds and diterpenoids. The LC-HRMS analysis confirmed the presence of Piperine and gallic acid as the major bioactive compounds in this formulation. The total phenolic content and total flavonoid content of this tablet were found to be 19.6 % and 2.08 % respectively. Total Antioxidant Activity of Vilwadi tablet was found to be 9.23 mg of ascorbic acid/ gram of extract. The formulation also exhibited good anti-inflammatory activity by inhibiting the denaturation of protein. Further, molecular docking studies showed that the major constituent of Vilwadi tablet, Piperine, has a greater binding affinity compared to the standard anti-inflammatory drug. The biological activities of Vilwadi tablet are due to the synergic effect of bioactive alkaloids, polyphenols and flavonoids present in it.
Keywords: *Anti-inflammatory activity; Gallic acid; Piperine; Vilwadi tablet.*

1. Introduction
Ayurvedic formulations have gained significant attention as valuable therapeutic agents as it is considered as a natural source of medicine. Their minimal adverse effects, affordability, and widespread availability have strengthened their role in healthcare. In recent years, polyherbal formulations have been increasingly employed for the management of various diseases due to their synergistic pharmacological effects. However, the enormous rise in the use of these natural medicines underscores a critical challenge in pharmaceutical research, which is the assurance of their quality, safety, and efficacy. Standardization of these herbal drugs is therefore indispensable for ensuring the quality of the product. Vilwadi tablet is an antitoxic ayurvedic formulation which is widely used to treat toxic effects and digestive disorders. The formulation is made up of thirteen medicinal plants and goat's urine. Studies have validated its therapeutic potential in managing skin diseases, including psoriatic lesions. Vilwadi tablet has been widely used during COVID-19 in managing moderate cases and as a preventive medicine (Divya et al., 2023). There remains a gap in the literature regarding the specific bioactive compounds responsible for the diverse pharmacological effects of Vilwadi tablet. Hence the present study mainly focused on the phytochemical analysis of Vilwadi tablet and evaluation of antioxidant and anti-inflammatory potential of the formulation [1].

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2. Method

2.1. Sample Collection and Extraction

Vilwadi Tablet was purchased from authorized Ayurvedic pharmaceutical company in Thrissur District, Kerala, India. The tablet was extracted with distilled water (10:1 v/w) at room temperature under stirring. The extract was filtered and made up to a known volume and kept under refrigeration [2].

2.2. Phytochemical Analysis

The water extract of Vilwadi tablet was tested for the presence of bioactive compounds by using standard methods (Yadav & Agarwala, 2011). The Total Phenolic Content in the water extract of Vilwadi tablet was determined by using Folin-Ciocalteau's (FC) reagent. Absorbance was measured at 760 nm using Shimadzu (UV 1800 240 V) UV-VIS spectrophotometer. The total flavonoid content in the sample was evaluated using quercetin as standard (Nampoothiri et al., 2011). The Total antioxidant activity was estimated using ammonium molybdate method (Prieto et al., 1999) [3].

2.3. LC-HRMS QTOF Analysis

The most important constituents of Vilwadi tablet were analysed by the LC-HRMS QTOF analysis using Agilent Mass Hunter software. Hypersil GOLD C18 100 x 2.1mm-3 MICRON was used as the column. Solvent system consisted of 0.1% formic acid in Milli-Q water and Acetonitrile [4].

2.4. Anti-Inflammatory Activity

(Protein Denaturation Inhibition Assay) The reaction mixture (0.5 ml) consisted of 0.4 ml bovine serum albumin (3% aqueous solution) and varying volume of test sample. The sample was incubated at 37ºC for 20 min and 2.5 ml phosphate buffered saline (pH 6.3) was added to each tube and then heated at 80ºC for 10 min. The absorbance was measured using UV–VIS Spectrophotometer (Thermo scientific, Orion Aquamate 8000) at 660nm [5].

2.4.1. Molecular docking

Computational tools have become very popular in the field of medicinal chemistry to predict the biological activities of different constituents based on structure-activity relationships. Molecular docking is a computational based drug design method to analyse the interactions between ligand and the target. The anti-inflammatory potentials of Vilwadi tablet were analysed by molecular docking studies using Autodock vina software. Piperine and gallic acid, the major phytochemicals in Vilwadi tablet, was taken for docking with COX-2 enzyme, which is the most common target for anti-inflammatory activity (Gupta, et al., 2022). Determination of active sites and visualization of molecular interactions between ligand and target enzyme was done by Biovia Discovery Studio [6].

2.5. Statistical Analysis

The experimental results were expressed as mean ± SD (standard deviation) of triplicate measurements. The data were subjected to one way analysis of variance (ANOVA) and the significance of differences between means were calculated by Duncan's multiple range test using SPSS for windows, standard version 7.5.1, SPSS and the significance accepted at $P \leq 0.05$ [7].

3. Results and Discussion

3.1. Phytochemical screening

The preliminary phytochemical screening of Vilwadi tablet indicates that it contains some important bioactive compounds such as polyphenols, flavonoids, alkaloids and terpenoids. The TPC and TFC of crude aqueous extract of Vilwadi tablet were found to be 19.6 ± 0.5% and 2.08 ± 0.32% respectively. In the TPC estimation, the active constituent in the FC reagent, is a mixture of phosphomolybdate and phosphotungstate, which oxidizes phenols in the sample to produce blue colour (Nampoothiri et al., 2012). Undesired effects of oxidative stress have been found to be controlled by the antioxidant activities of these phenolic compounds (Pérez et al., 2023). Vilwadi tablet showed a significant total antioxidant activity by reducing the ammonium molybdate and was found to be 9.23 ± 0.25 mg of ASC/gram of extract which indicates the ability of the tablet to scavenge the free radicals and convert it into neutral species [8].

3.2. The LC-HRMS Profiling of Vilwadi Tablet

Different bioactive substances such as alkaloids, flavonoids and polyphenolic compounds were identified from the LC-HRMS profiling of Vilwadi tablet in both positive and negative modes of ionization (figure 1 and 2). In the negative ionization mode, a major peak at 169.014 indicates the presence

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of gallic acid. Gallic acid is a polyphenolic compound which has been used as a healing agent for centuries. It helps in protecting against the harmful effects of UV radiation. It is also found to have anti-cancer, anti-HIV, antiulcer and antimicrobial properties (Wianowska & Olszowy-Tomczyk, 2023). Further, a peak at 286.14 indicates another major compound Piperine (positive ionization mode) which is an important alkaloid with good pharmacological actions such as antiproliferative, antitumor, antioxidant, antidiabetic, antiobesity, cardioprotective, antimicrobial, antiaging, immunomodulatory, hepatoprotective, anti-allergic, anti-inflammatory, and neuroprotective properties (Haq et al., 2021) and it is the major constituent of Piper nigrum and Piper longum, both of which are used in the preparation of Vilwadi tablet. Besides these, various other bioactive compounds such as Quinic acid (QA), Caffeic acid (CAF), Norbelladine (NOR), L-malic acid (MA), Kokusaginine (KOK), and Atropine (ATR) were also present in minor amounts. These chemical constituents are reported for the first time on Vilwadi tablet [9].

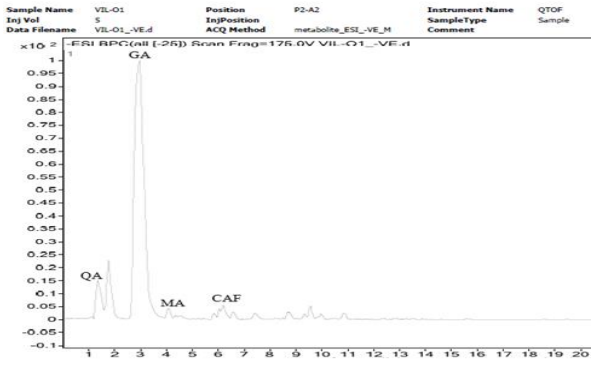

Figure 1 LC-HRMS QTOF Analysis (-ESI)

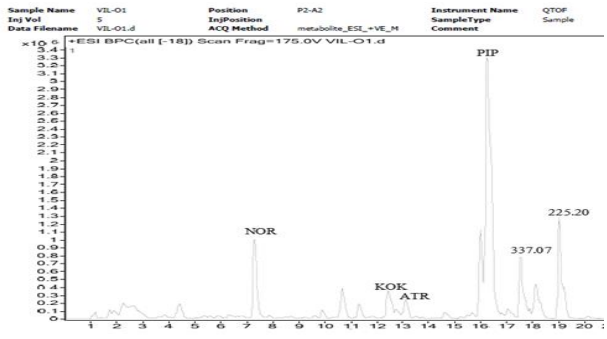

Figure 2 LC-HRMS QTOF Analysis (+ESI)

Table 1 indicates the bioactive compounds identified in Vilwadi tablet with their retention time and m/z values.

Table 1 Bioactive Compounds Identified in Vilwadi Tablet

Compound	RT	m/z
Quinic acid (QA)	1.33	191.05
Gallic acid (GA)	2.966	169.014
L-malic acid (MA)	4.501	133.01
Caffeic acid (CAF)	6.596	179.03
Norbelladine (NOR)	7.984	260.12
Kokusaginine (KOK)	12.419	260.09
Atropine (ATR)	13.053	290.17
Piperine (PIP)	16.222	286.14

3.3. Anti-inflammatory Activity

Vilwadi tablet showed good anti-inflammatory activity (IC50 3320.1µg) by inhibiting protein denaturation. The protein denaturation assay is based on the idea that substances with anti-inflammatory properties may be able to stabilize protein structures and prevent denaturation, which reduces inflammation and tissue damage. The synthetic anti-inflammatory drugs can cause serious side effects such as gastro-intestinal irritation, cardiovascular problems, drug dependency, thymus suppression, anaemia (Gupta et al., 2022). Hence it is better to choose plant derived drugs even though their efficacy is lower than the standard anti-inflammatory drug Diclofenac (IC50 49.68µg). Currently, the biological activities of isolated compounds from medicinal plants are assessing through molecular docking (Rajeswari et al., 2020). The major bioactive constituent of Vilwadi tablet, Piperine, exhibited significant anti-inflammatory potential in molecular docking analysis against COX-2 as the target (figure 3 & 4). A more negative docking score indicates a strong interaction between the ligand and protein. Here it was observed that Piperine has a greater anti-inflammatory activity with a docking score of -8.9Kcal/mol compared to the standard drug Diclofenac (-8.1 Kcal/mol). This suggests that Piperine may be a key contributor to the anti-inflammatory activity of Vilwadi tablet, owing to its

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high abundance in the formulation. Molecular docking of gallic acid, which is also a major compound of Vilwadi tablet, was also performed against COX-2 which showed a binding affinity of -6.9 Kcal/mol which is lower than that of Diclofenac [10].

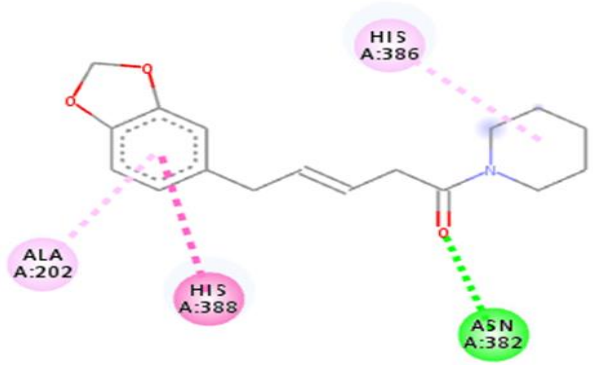

Figure 3 Docking Studies of Piperine with COX-2

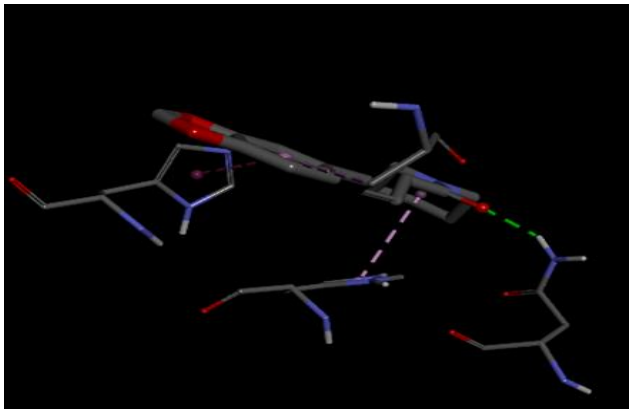

Figure 4 Ligand Interaction in Docking Studies

Conclusion

LC-HRMS analysis of Vilwadi tablet revealed the presence of several key bioactive compounds, notably, Piperine and gallic acid as the major compounds. These constituents are known for their potent antioxidants and pharmacological action, aligning with the traditional use of formulation. The biological evaluations suggest that the formulation exhibits significant antioxidant and anti-inflammatory activities. Molecular docking studies indicate that the major compound in Vilwadi tablet, Piperine, has a significant affinity for inflammatory targets, supporting its potential role in the tablet's anti-inflammatory efficacy.

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