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# PHYTOCHEMICAL SCREENING OF SECONDARY METABOLITES PRESENT IN *WOODFORDIA FRUTICOSA* LEAVES AND THEIR ANTIBACTERIAL PROPERTIES WITH DIFFERENT SOLVENT EXTRACTS

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## ABSTRACT

The present study to characterize the *Woodfordia fruticosa* for the presence of biologically active phytochemicals, antioxidant properties and antibacterial properties using various solvents extracts of leaves it was found that all the screened metabolites were present in higher amount in their respective effective solvents confirmed by TLC. Here Chloroform (for alkaloid and terpenoid), Methanol (for flavonoid and tannin) and aqueous extract (for cardiac glycoside and saponin) were identified as effective solvents for these metabolites. Tannin percentage found to be higher (1.496%) whereas antioxidant property was poor (1.14% glutathione) in leaves of *Woodfordia fruticosa* as compare to *Catharanthus roseus* with value (0.693%) for tannin and 25.09  $\mu$ mole/ml glutathione for its antioxidant property. Antimicrobial assay showed that methanolic extracts were more effective than other solvent extracts with (14mm, 19mm, 17mm, and 11mm) zone of inhibition against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* respectively. The results obtained in this study confirmed the presence of important therapeutically compounds in the leaf extracts predominantly tannins and methanolic extracts was identified as an effective solvent for both phytochemical estimation and antibacterial property of *Woodfordia fruticosa* and would be used as better option for future drugs discoveries to cure various diseases.

## INTRODUCTION

India has a rich habitat of medicinal plant which used as traditional medicine from thousands of years (Choudhury *et al.*, 2012) and also in rural areas of the many developing countries (Sandhu and Heinrich, 2005) and for commercial drugs both for use in preventive and curative medicine in the treatment of infectious disease (Saranraj and Sujitha, 2015). Infectious diseases are the world's leading cause of premature death and caused by variety of bacteriological agents including *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* which is most common (Paramar and Rawat, 2012; Bhattarai and Bhuju, 2011). In recent years, drug resistance to human pathogenic bacteria has been commonly reported from all over the world. Antibiotics not only have adverse effects on host but also its continuous use develops resistance in micro organism which demands to develop alternative antimicrobial drugs for the treatment of infectious diseases (Manandhar, 2002). The curative properties of medicinal plants are perhaps due to the presence of various secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, saponins, sterols etc. Dhawai/*Woodfordia fruticosa* is spreading evergreen shrub belongs to the family lythraceae. It is native of Asia and Africa and it is widely distributed in throughout the world (Thakur *et al.*, 1989). Many organic constituents like tannins, phenols, steroids/terpenoids, carbohydrates, resins and inorganic ones including iron, aluminum, calcium, magnesium, potassium etc., have been present in this plant. Traditionally it's all part is useful, but it is generally cultivated for its flower and leaves. It is act like Stimulant, astringent, Tonic and used in Diarrhea, Dysentery, Fever, Headache, Ulcer and wound (Grover and Patni, 2013; Chadha, 1976). According to an estimate, approximately plant based drugs prescribed for use through the world come from just 95 plant species (Lewington, 1990). The shortcomings of the drugs available today propel the discovery of new pharmacotherapeutic agents from medicinal plant research (Cordell, 1993).

Thus, the preliminary phytochemical screening is of paramount importance in identifying new source of therapeutically and industrially valuable compound having medicinal significance, to make the best and judicious use of available natural wealth. As of my knowledge very few works had been done to investigate phytochemical properties of *Woodfordia fruticosa*. Knowing the ethno botanical and pharmacological applications of the plant and in order to promote the use of *Woodfordia fruticosa* as herbal medicines the objective of this research is to assess its phytochemical investigation of secondary metabolites.

## MATERIALS AND METHODS

Well developed plant of *Woodfordia fruticosa* collected from Garden of Universal Medicaments Pvt. Ltd, Shanti Nagar, Nagpur (M.H.). Leaves of this plant was dried in sun light for 3 days. The present study was conducted at Unijules life science Pvt. Ltd. Nagpur.

### Methods

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### Extraction of secondary metabolite from *Woodfordia fruticosa* leaves extracts

Extraction was done in the Soxhlet extractor by 5 g dried leaves of *Woodfordia fruticosa*. About 80-90 ml of each of the solvent was taken and the leaves were repeatedly extracted for 18 hrs with Hexane (200mL x 2) and subsequently with Chloroform, Methanol and Distilled water/Aqueous extract (200mL x 2) [Precaution should be taken, that continuous flow of water is maintained].

### Phytochemical screening for secondary metabolites identification

The phytochemical screening of secondary metabolites present in *Woodfordia fruticosa* leaves extracts were done with these four solvents. The extracts of these solvents were screened for different classes of secondary metabolites using following biochemical test as suggested by Harborne (1998) and Parekh and Chanda (2007).

#### Test for alkaloids

1 mL of extract was heated on boiling water bath with 2N HCL (5mL). After cooling the mixture was filtered and the filtrate was treated with few drops of Wagner's Reagent. The samples were observed for turbidity or flocculation, the extent of turbidity obtained determines the amount of alkaloid (Salehi and Surmaghi *et al.*, 1992).

#### Test for cardiac glycosides

0.5mL of extract was treated with 0.2ml of glacial acetic acid containing 1 drop of  $\text{FeCl}_3$ . This was under layered with 1 ml of  $\text{H}_2\text{SO}_4$ . A brown ring formed at the interface indicates presence of cardiac glycosides, the size of the ring determines the amount of cardiac glycosides (Ajaiyeobu, 2002).

#### Test for flavonoid

To a portion of the filtrate 5ml of dilute ammonia was added followed by addition of concentrated  $\text{H}_2\text{SO}_4$ . A yellow coloration observed in each extract indicated the presence of flavonoid, the extent of coloration obtained determines the amount of flavonoid (Sofowara, 1993).

#### Test for saponins

The extract is dissolved in water and shaken well. If froth formation is seen it indicates presence of saponins, the amount of froth obtained determines the amount of Saponins (Kapoor *et al.*, 1969).

#### Test for tannins

5ml of extract was taken in a test tube and to it few drops of 0.1%  $\text{FeCl}_3$  solution was added, a brownish green or blue

black precipitation indicates presence of tannins, the amount of precipitation obtained determines the amount of Saponins (Segelman *et al.*, 1969).

#### Test for terpenoids

5ml of extract was taken in a test tube and was mixed with 2ml of chloroform and 3ml of concentrated  $\text{H}_2\text{SO}_4$ . A reddish brown color at the interface indicates presence of terpenoids (Evans, 1997).

#### Confirmation of secondary metabolites by TLC

The preliminary phytochemical analysis for conformation of these metabolites was also carried out by using thin layer chromatography (TLC) as suggested by (Waksmundzka-Hajnos *et al.*, 2008 and Harborne, 1998) with effective solvent extracts identified by phytochemical screening. Details of mobile phase and spraying reagents are presented in Table 2.

#### Quantification of tannin in *Woodfordia fruticosa* leaves

The percentage amount of tannin present in *Woodfordia fruticosa* plant leaves was quantified by using Lowenthal Permanganate titration method (Lowenthal, 1877; Rajpal, 2002) as following: Take 1 gm of sample in 100 ml of water, filter, transfer 10 mL of filtrate to a conical flask 1 litre capacity, add 750 mL of water and 25 mL of indigosulphonic acid solution and titrate with constant stirring against N/10  $\text{KMnO}_4$  to a golden yellow color end point. A blank was carried out simultaneously titrating 25 mL of indigo sulphonic acid in 750 ml of water.

**Here** Each mL of N/10  $\text{KMnO}_4$  = 0.004157 gm of Tannins.

#### Estimation of glutathione in leaves of *Woodfordia fruticosa*

The antioxidant property of *Woodfordia fruticosa* was estimated by using glutathione estimation in triplicates by using following steps as suggested by Eyer and Podhradsky (1986) and Rotruck *et al.* (1973). The reaction mixture consisting of 0.8 ml of 0.4 M sodium phosphate buffer (pH 7.0), 0.2 ml of 10 mM sodium azide (65 mg/100 mL water), 0.4 ml of 4 mM reduced glutathione (GSH), 0.2 mL of 2.5 mM  $\text{H}_2\text{O}_2$ , 0.4 ml of water and 1 ml of sample was incubated at 90 seconds. The reaction was terminated with 1 mL of 10% trichloroacetic acid (TCA) and after centrifugation (3000 rpm for 10 minutes); 2 mL of the supernatant was added to 3 ml of phosphate buffer and 1ml of DTNB (5,5,2 -dithiobis-(2-nitrobenzoic acid) reagent (0.04% DTNB in 1% sodium citrate). The color developed was read at 412 nm and the enzyme activity is expressed in terms of  $\mu\text{M}/\text{mg}$ . Assay was done 4 times and the result was presented in the form of mean value.

**Here** Amount of TNB (2-nitro-5-thiobenzoate) produced =



Figure 1: Different solvents extract of *Woodfordia fruticosa* leaves

Amount of Glutathione in plant leaf extract

### Antibacterial Assay of *Woodfordia fruticosa* Leaves Extracts

#### Bacterial strain used

The bacterial strains used to access the antibacterial properties of different crude solvent extract of *Woodfordia fruticosa* leaves were Gram-positive *Staphylococcus aureus*, *Bacillus subtilis* and Gram-negative *Escherichia coli*, *Pseudomonas aeruginosa*. The investigated microbial strains were obtained from Unijules life sciences, Laboratory, Nagpur, India. The organisms were maintained on nutrient agar (Hi Media, India) slope at 4°C and activated by sub culturing.

#### Antibacterial assay

Antibacterial activity of the crude extracts in different solvents was tested by disc diffusion assay (Taylor *et al.*, 1995). Mueller Hinton agar no. 2 (Hi Media, India) was used as the bacteriological medium. Medium was prepared and poured 20 mL each in sterilized Petri plates of 9 cm diameter and allowed to solidify. Bacterial cultures grown in nutrient broth and on agar slants were used. Bacterial suspension was prepared aseptically from 10 mL of saline (0.085 g NaCl in 10 ml Distilled water) under laminar. The plates, cultured with microbial suspension (100- 150 µL) by spread plate technique. The zone of inhibition was measured after 24 hrs using disc diffusion assay. The 40 µL of each extracts were used for antibacterial assay. For each bacterial strain controls were maintained where extract free pure solvents were used. The control zones were subtracted from the test zones and the resulting zone diameter is shown in the Table 3. The experiments were performed twice and presented as mean values.

## RESULTS AND DISCUSSION

The present study was done under the objectives of extraction, screening, TLC, Antioxidant test, quantification of Tannin, and antibacterial assay of *Woodfordia fruticosa* leaves. The results of above objectives are as follow:

#### Different solvent extracts of *Woodfordia fruticosa* leaves

On the basis of visual observation of different solvent extracts of *Woodfordia fruticosa* leaves showed that concentration of crude extracts was highest in methanolic extracts followed by distilled water extracts whereas lowest in hexane extracts (Fig. 1). Earlier findings as reported by (Grover *et al.*, 2014; Abegunde and Ayodele-Oduola, 2013) also find that methanolic extracts was better solvent for secondary metabolites extraction. By this result we can say that methanol

should be better solvent for secondary metabolites present in *Woodfordia fruticosa*.

#### Phytochemical screening for secondary metabolite:

Preliminary qualitative test is useful in the detection of bioactive principles and subsequently may lead to drug discovery and development (Mallikarjuna *et al.*, 2007) and also an increasing interest in the phytochemical compounds, which could be relevant to their nutritional incidence and their role in health and disease (Steinmetz and potter, 1991). Moreover, a plant extract may contain several thousand different secondary metabolites but phytochemical analysis will reveal only a narrow spectrum of its constituents (Grover *et al.*, 2014). In the present study screening the extracts with phytochemical test for secondary metabolites present in *Woodfordia fruticosa* leaves leaves, it was found that this plant gives both positive and negative results with different solvents extracts by conformational changes (Table 1).

Here the solvent extracts which showed higher amount of conformational changes termed as effective solvents and would be used for TLC conformation for these secondary metabolites. Table 1 showed that Chloroform leaf extracts had higher amount of alkaloid, methanol extracts has higher amount of flavonoid and tannin, aqueous extracts had higher amount of cardiac glycosides and saponins while hexane extracts was not identified as an effective solvents for any of the metabolites. Hence chloroform is an effective solvent for alkaloid and terpenoid extraction, Methanol is an effective solvent for flavonoid and tannin extraction and water is an effective solvent for cardiac glycosides and saponins extraction from *Woodfordia fruticosa* leaves. The previous study by Grover *et al.* (2014) also confirms the presence of these metabolites through phytochemical screening and found that *Woodfordia fruticosa* leaves contain predominantly tannin and terpenoids. Dubey *et al.* (2014) also confirmed the presence of these metabolites along with other metabolites by mass spectrometry analysis. These results indicate that *Woodfordia fruticosa* plant is a good source of phenolics and other metabolites which support its use in most of the regions where people consume this herb as a herbal medicines for various purposes.

#### TLC Confirmation of Secondary Metabolites in *Woodfordia Fruticosa* Leaves

The chromatographic and spectroscopic techniques have proved very useful in isolation and proper identification of the active constituents in the plant extracts (Kataria *et al.*, 2011). Thin layer chromatography is the widely used analytical tool in herbal drug standardization process due to its simplicity and cost effectiveness (Aman *et al.*, 2008).

**Table 1: Phytochemical Screening of *Woodfordia fruticosa* leaves for Secondary Metabolites**

| Secondary metabolites/extracts | Conformational changes   | Chloroform extracts | Methanol extracts | Aqueous extracts | Hexane extracts | Effective solvent |
|--------------------------------|--------------------------|---------------------|-------------------|------------------|-----------------|-------------------|
| Alkaloid                       | Yellow with turbidity    | ++                  | +                 | -                | -               | Chloroform        |
| Cardiac glycosides             | Brown ring               | -                   | ++                | +++              | -               | Aqueous           |
| Flavonoids                     | Yellowness               | +                   | ++                | +                | -               | Methanol          |
| Saponins                       | Foam                     | +                   | -                 | ++               | -               | Aqueous           |
| Tannin                         | Blue black Precipitation | ++                  | +++               | +                | +               | Methanol          |
| Terpenoids                     | Brown ring               | ++                  | +                 | -                | -               | Chloroform        |

Where, +++ = present in higher concentration; ++ = present in medium concentration; + = present in lower concentration; - = absent

Table 2: TLC confirmation of secondary metabolites presents in *Woodfordia fruticosa* leaves extracts with different mobile phase and spraying agents

| Secondary metabolites | Effective solvent Extracts | Mobile phase                                  | Spraying reagents                                     | No. of spots | Spot Color      | RF value |
|-----------------------|----------------------------|---|---|--------------|-----------------|----------|
| Alkaloids             | chloroform                 | Butanol: Glacial Acetic acid: Water (10:10:5) | Zink chloride   | 1            | pink            | 0.9500   |
| Cardiac glycosides    | Distilled water            | (chloroform: methanol: water (80:19:1)        | Chloramine-trichloroacetic acid                       | 1            | Yellowish brown | 0.1000   |
| Flavanoids            | methanol                   | n-Butanol: Acetic acid: Water (6:1:2)         | Bromocresol Green                                     | 1            | Light green     | 0.9482   |
| Saponins              | Distilled water            | Chloroform: methanol: Water (6.5:3.5:1.0)     | Anisaldehyde, glacial acetic acid and conc. $H_2SO_4$ | 1            | yellow          | 0.1338   |
| Tannins               | methanol                   | Butanol: Acetic Acid: water (6:2:2)           | Ferric chloride                                       | 1            | black           | 0.9500   |
| Terpenoids            | chloroform                 | Toluene: Ethyl acetate: Water (8.5:1.5)       | Anisaldehyde - Sulphuric Acid                         | 1            | brown           | 0.9615   |

TLC profiling of plant extracts have resulted in directing towards the presence of a number of phytochemicals. Various phytochemicals have different R<sub>f</sub> (Retention factor) values in different solvent system (Grover *et al.*, 2014). Here TLC results confirmed the presence of important metabolites (alkaloids, terpenoids, tannins, saponins, and flavonoid) in *Woodfordia fruticosa* plant leaves in their effective solvent extracts. The R<sub>f</sub> value was found ~ same as compare to reference for the particular metabolites illustrate in table 2. Similar results also observed by Grover *et al.*, (2014) in *Woodfordia fruticosa* leaves where they confirm the presence of tannin, flavonoid and other metabolites.

#### Glutathione assay

Natural antioxidant has been given more attention due to protective effects against drug-induced toxicity studies especially whenever free radical generation is involved (Ramadoss *et al.*, 2012). Here in the present study the value obtained in the antioxidant test of *Woodfordia fruticosa* leaves extracts by Glutathione assay tells that amount of glutathione in *Woodfordia fruticosa* leaves extracts is very less (1.14  $\mu$ mole/ml) as compare to known highly antioxidant plant leaves of *Catharanthus roseus* with value 25.09  $\mu$ mole/ml. So the steps for extracting and also increasing the glutathione content from this plant should be developed. Previously Finos *et al.*, (2011); Bhatt and Baek, (2005) also revealed antioxidant property of *Woodfordia fruticosa* flower by DPPH assay in methanol, chloroform and petroleum ether extracts. This result indicate that *Woodfordia fruticosa* leaves extracts has less effective antioxidant as compare to its flower so that its leaves is not a effective agents to anticancerous drug development.

#### Quantification of tannin

Tannins and tannin-like substances are widespread in nature and play important role in prevention of chronic diseases. It exerts anti-inflammatory, antimicrobial, antioxidant, anti-carcinogenic and body mass reducing activities (Saxena *et al.*, 2013). Herbs that have tannins as their main component are astringent in nature and are used for treating intestinal disorders such as diarrhea and dysentery (Subhuti, 2003). The quantification of tannin showed that tannin percentage (w/w of tannic acid) in 1 gm sample of *Woodfordia fruticosa* leaf was higher (1.496 %) as compare to *Catharanthus roseus* (0.693 %). Previously Dubey *et al.*, (2014); Khan *et al.*, 2011 also showed agreements with our findings. The Tannins fraction have high medical activities thus steps need to be developed to increase the tannin content of the plant and also good extraction steps for extracting tannins will be required.

#### Antibacterial assay

Every culture throughout the world has been using herbal and natural products of folk medicine from centuries. Various plant parts such as leaves, bark, fruits, roots and seeds are used in treatment of various diseases (Kumar *et al.*, 2013). The importance of *Woodfordia fruticosa*/Dhawai in the community can perhaps be understood by the broad spectrum of its antibacterial activity. In the present study of antibacterial activity of *Woodfordia fruticosa* leaves extracts from different solvent showed that out of four solvent leaves extracts methanol, chloroform and aqueous extracts showed the zone of inhibition against four bacterial strains whereas hexane extracts showed

**Table 3: antibacterial assay of different extracts of Dhawai and Sadabahar**

| Microbial strains   | Diameter of Clear Zone (mm) |                    |                  |                  |
|---------------------|-----------------------------|--------------------|------------------|------------------|
|                     | HexaneExtracts              | ChloroformExtracts | MethanolExtracts | Aqueous Extracts |
| <i>B.subtilis</i>   | -                           | 13                 | 14               | 9                |
| <i>S.aureus:</i>    | -                           | 13                 | 19               | 5                |
| <i>E.coli</i>       | -                           | 14                 | 17               | 8                |
| <i>P.aeruginosa</i> | -                           | 11                 | 11               | 7                |

no zone of inhibition against these microorganisms.

Here methanolic extracts were more effective than other solvent extracts with (14mm, 19mm, 17mm and 11mm) zone of inhibition followed by and chloroform extracts of with (13mm, 13mm, 14mm and 11mm) zone of inhibition against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* respectively. The highest zone of inhibition (19 mm) obtained in the methanolic extracts against *Staphylococcus aureus* followed by *Escherichia coli* (17 mm) whereas lowest (5mm) found in aqueous extracts against *Staphylococcus aureus* (Table 3).

From the above result we can say that hexane extract of *Woodfordia fruticosa* leaves was found to be not effective on the microbial strains whereas methanol extract was found to be more effective followed by chloroform and aqueous extracts. The present study of Dhawai showed agreement with the results of Bhattarai and Bhaju, (2011) and partial agreement with the Dubey et al., (2015) in which they found antibacterial activity of methanolic, chloroform and aqueous extracts along with hexane extracts of *Woodfordia fruticosa* leaves. The present results of antimicrobial activity and its conformation with the previously reported work we can say that *Woodfordia fruticosa* leaves extract should be used as potent inhibitors of infectious bacterial organism and used as antimicrobial drug for infectious diseases. We hope that the results of antimicrobial activities may play a significant role in the conservation of traditional medicine knowledge of *Woodfordia fruticosa* and encourage the scientific community for further investigations of antimicrobial activity will be required to see the effectiveness of these extracts against other infectious microbial agents.

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