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Received: 14-03-2017 Accepted: 29-05-2017 DOI: 10.18805/asd.v37i2.7983

ABSTRACT
Present study aims to investigate phytochemical analysis as well as in vitro antimicrobial activity of crude aqueous, methanol, ethanol, chloroform and petroleum ether extracts from leaves of C. roseus. Antimicrobial activity of extract was studied against various bacterial strains (Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus and Mycobacterium smegmatis). Qualitative preliminary phytochemical analysis revealed that alkaloids, phenols, flavonoids, amino acid and cardiac glycosides are present in the extracts. The result for total phenol and flavonoids content was the highest in methanol and the lowest in petroleum ether crude extract. The study revealed that inhibition significantly depend upon the solvent used for extraction and bacterial strain tested for susceptibility. Aqueous extracts were found less efficient as compared to organic solvent. Methanolic extract were found more effective against tested microbes.

Key words: Anti-bacterial activity, Catharanthus roseus, Flavonoids content, Phytochemical analysis, Total phenols.

INTRODUCTION
Medicinal plants are effective for treating as well as for management of many intractable human diseases because they contain several phytochemical constituents. Medicinal plants are considered as natural chemical factories for synthesis of chemical agents with therapeutic and safer and cheapest sources of bioactive compounds that can produce a definite effect physiologically on human system properties (Gobalakrishnan et al., 2013).

These compounds incorporate with primary and secondary metabolism of plants and are present in all parts like root, stem, bark, leaves, flowers and fruits or plant exudates (Mathew et al., 2012). These secondary metabolites vary significantly in their quality and quantity in different plant parts (Vijayalakshmi et al., 2014). Several studies have revealed that the secondary metabolites exhibit certain important pharmacological activities viz. anti-bacterial, anti-fungal, anti-cancer, anti-malarial, anti-viral, anti-diabetics and anti-inflammatory (Islam et al. 2010). Emergence of infectious diseases and antibiotic resistant bacterial strains pace the discovery of alternate novel antimicrobial therapeutic agents from medicinal plants (Omwenga et al., 2009). Even though ever rising advancement in field of molecular diagnosis and medicine, it is estimated that approximate 80% world population from developing countries still depends on natural products derived from medicinal plants.
evaluation of crude leaf extracts of *Catharanthus roseus* in aqueous, methanol, ethanol, chloroform and petroleum ether for phytochemical analysis and *in vitro* anti-bacterial activity against (*Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Mycobacterium smegmatis*).

**MATERIALS AND METHODS**

The present investigation was carried out at Department of Botany, Punjabi University, Patiala (Punjab) during 2014-15, to study *in vitro* anti-bacterial activity and phytochemical screening of crude extracts of *Catharanthus roseus*.

**Plant materials:** The present study focused on plant which was *Catharanthus roseus*. Chemicals methanol, ethanol, H$_2$SO$_4$, acetone, Wagner’s reagent (iodine in potassium iodide), NaOH, HCl, FeCl$_3$, chloroform, glacial acetic acid, tannic acid, quercetin and ninhydrin.

**Sample collection:** Plants leaves were collected from the conservatory of Punjabi University, Patiala. Fresh and tender leaves of selected plants were used for phytochemical analysis.

**Microorganisms:** Reference bacterial strains were obtained from the Botany Department, Punjabi University, which included *E. coli*, *B. subtilis*, *P. aeruginosa*, *S. aureus* and *M. smegmatis*. The strains were kept at 4°C on agar slant for further any test.

**Preparation of plant extract and phytochemical screening:** The leaves of the chosen plants was collected and then washed under running tap water followed by rinse using distilled water for the removal of dust and soil particles. The leaf samples were then dried under shade at room temperature and crushed to fine powder and kept in air tight polythene bags for future use (Susmit and Archana, 2011).

**Solvent extract of sample:** The extracts of sample were prepared by soaking 5 g of dried leaf powder in 50 mL of each; double distilled water, methanol, ethanol, chloroform and petroleum ether and shaken well. The solution was left at room temperature for 72 hours and then filtered with the help of sterilized Whatman filter paper. The filtrate of the selected plant samples were taken and used for further phytochemical analysis.

**Photochemical screening**

**Test for Alkaloids (Wagner’s reagent):** Extracts (2-3 mL) were treated with approximately 1 mL of Wagner’s reagent (1.27 g of iodine and 2 g of potassium iodide in 100 mL of water) and observed for the formation of deep blue or black colouration, which confirms the presence of phenols (Hema et al., 2012). 

**Test for carbohydrates (Fehling’s Test):** Fehling A and B reagents were mixed with 2 mL of extract and boiled in water bath for 10 minutes. Precipitates of cuprous oxide were formed, if reducing sugars were present, which were brick red in colour.

**Test for cardiac glycosides (Keller Kelliani’s test):** Extracts (2 mL) were treated with glacial acetic acid with few drops of 5% ferric chloride solution, carefully under laid with 1 mL concentrated H$_2$SO$_4$. Reddish brown ring at the interface indicated the presence of cardiac glycosides (Kumar et al., 2013).

**Test for flavonoids (Alkaline reagent test):** Extract (2mL) was treated with few drops of 20% sodium hydroxide solution acute yellow colouration which disappeared on addition of dilute hydrochloric acid, indicates the presence of flavonoids (Khandewal, 2008).

**Test for phenols (Ferric chloride test):** Extracts (2 mL) were treated with 0.5 mL of aqueous 5% ferric chloride and observed for formation of deep blue or black colouration, which confirms the presence of phenols (Hema et al., 2012).

**Test for phlobatannins (Precipitate test):** Extract (2 mL) was boiled with 1mL of 1% HCl, deposition of a red precipitate showed the presence of phlobatannins (Ayoola et al., 2008).

**Test for saponins (Foam test):** Distilled water (6 mL) was added in 2 mL of extract. The mixture was shaken thoroughly in graduated cylinders for 15 minutes and observed for the formation of constant foam to confirm the presence of saponins (Dubey and Sushma, 2014).

**Test for tannins (Braymer’s test):** Ferric chloride solution (10%) was added to 2 mL of extract and observed for formation of blue or greenish coloured solution.

**Test for terpenoids (Salkowki’s test):** Chloroform (2 mL) was added to 2 mL of extract and added a few drops of concentrated H$_2$SO$_4$. The mixture was shaken well. A reddish brown precipitate produced immediately indicates the presence of terpenoids (Mir et al., 2013).

**Test for quinoes:** Extracts (2 mL) were added with a few drops of concentrated HCl; formation of yellow precipitate (or colouration) indicates the presence of quinoes (Ugochukwu et al., 2013).

**Estimation of total phenolic content:** The total phenolic content (TPC) was estimated by spectrophotometer using Folin-Ciocalteu method (Singleton and Rossi, 1965). Folin-Ciocalteu’s reagent (5mL) (1:10 diluted) was added with 200 µL of diluted sample. Then 4 mL of 7% sodium carbonate solution was added after 4 minutes. The mixture was mixed thoroughly by vortex for 2 minutes and then kept at 40°C for 30 minutes, after which the absorbance was
measured at 765 nm. The TPC was estimated by using tannic acid (0.02–0.1 mg/mL) as a standard calibration curve. The results were expressed as milligrams of tannic acid equivalent (TAE) per gram of dried plant sample.

**Estimation of total flavonoid content:** Flavonoids content was predicted by the aluminum chloride method (Park et al., 2008). Extract (0.3mL), 0.15 mL of NaNO₂, (0.5 M), 3.4 mL of 30% methanol and 0.15 mL of 0.3 M AlCl₃·6H₂O were taken in a test tube. Then 1 mL of 1M NaOH was added after 5 minutes. The mixture was thoroughly mixed and absorbance was recorded against the blank reagent at 506 nm. Quercetin was used as a standard solution (0 to 100 mg/L) to obtain standard curve. The total flavonoids were expressed as milligrams of quercetin equivalents per gram of dried plant sample.

**Anti-bacterial assay:** Twenty five grams of nutrient agar (Sigma-Aldrich, Germany) was dissolved in one liter of distilled water and boiled. Nutrient agar was then autoclaved for 15 minutes at 121°C and left to cool at room temperature. Once the medium was cooled, it was poured into petridish. Each petridish was left for 30-40 minutes till solidification. Agar well diffusion method was used for anti-bacterial assay. Overnight grown culture (25µL) was spread onto 20 mL of sterile agar plates by using a sterile L-shaped spreader and the surface was allowed to dry for about 5 minutes. Wells were punched over the agar plates using sterile gel puncher and the extracts were added at various concentrations (50, 75 and 100 mg/100 mL of DMSO). 100 µL of different test extracts were added to the well. Chloramphenicol 2 mg/10 mL of DMSO was used as positive control while DMSO was used as negative control. The plates were incubated for 24 hours at 37°C. The diameter of inhibitory zones formed around each well were measured in mm and recorded.

**Statistical analysis:** Data were analyzed using CPCS-1 software (Gomez and Gomez, 1984). The CPCS-1 procedure was used to analyze variance and to determine statistical significance of treatment effects with one way analysis of variance (ANOVA). An ANOVA was performed according to the randomized block design layout with three replicates. The results were regarded as statistically significant.

**RESULTS AND DISCUSSIONS**

The current investigation was carried out for phytochemical analysis and anti-bacterial assay of the *C. roseus* (Figure 1) by using different organic and aqueous solvents for susceptibility test against the five bacterial strains (*E. coli, B. subtilis, P. aeruginosa, S. aureus* and *M. smegmatis*).

Results obtained from qualitative phytochemical analysis of the crude leaf extracts of *C. roseus* are presented in (Table 1). Of the 11 phytochemicals analyzed, 9 were present in various solvent extracts, viz. alkaloids, flavonoids, phenols, carbohydrates, saponins, quinones, tannins and cardiac glycosides. Only two phytochemical constituents viz. terpenoids and phlobatannins, were absent in all extracts. Alkaloids and cardiac glycosides were present in each extract and amino acids, saponins, flavonoids and quinones were present only in aqueous extract. Phenols and tannins were found in ethanol, methanol and aqueous extracts. The result of the analysis in various solvent has shown a remarkable variation in the presence of phytochemical compounds.

The total phenol contents (TPC) of five crude extracts determined by Folin-Ciocalteu method were recorded as tannic acid equivalents. The TPC in the different crude extracts varied from 8.1 to 40.8 mg TAE/g dried plant sample. Among the five crude extracts, methanol extract contained the highest (40.8 mg/ g) amount of phenol compounds followed by ethanol extract (23.3 mg/g), aqueous extract (12.9 mg/g), chloroform extract (10.54 mg/g), and petroleum ether (8.1 mg/g) (Table 2). The result of total flavonoids contents (TFC) of the five crude extracts of *C. roseus* is given in (Table 3). The TFC in the different crude extracts varied from 4.21 to 14.3 mg quercetin/g dried plant sample. Among the five crude extracts, methanol extract contained the highest (14.30 mg/g) amount of flavonoid compounds followed by ethanol (12.0 mg/g), aqueous (9.50 mg/g), chloroform (7.80 mg/g) and petroleum ether (4.21 mg/g). In previous studies on phenolic and flavonoids content, similar trend has been reported by Aligiani et al., 2001. The range in the observation of TPC

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**Table-1:** Phytochemical analysis of different extracts of *C. roseus*.

<table>
<thead>
<tr>
<th>Phytochemical test</th>
<th>Aqueous</th>
<th>Methanol</th>
<th>Ethanol</th>
<th>Chloroform</th>
<th>Petroleum ether</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloid</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Amino-acid</td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phenol</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Tannin</td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cardiac glycosides</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Terpenoids</td>
<td>+</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Quinones</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phlobatannins</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>
Table-2: Total Phenolic content mg tannic acid equivalent per g of leaves of C. roseus

<table>
<thead>
<tr>
<th>Solvent</th>
<th>TPC (mg/g) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>12.9±0.430</td>
</tr>
<tr>
<td>Methanol</td>
<td>40.8±1.02</td>
</tr>
<tr>
<td>Ethanol</td>
<td>23.2±0.722</td>
</tr>
<tr>
<td>Chloroform</td>
<td>10.54±0.430</td>
</tr>
<tr>
<td>Petroleum ether</td>
<td>8.1±0.523</td>
</tr>
</tbody>
</table>

Values are the average of triplicate experiments and values expressed as mean ± SEM.

Table-3: Total Flavonoids content mg quercetin equivalent per g of leaves of C. Roseus

<table>
<thead>
<tr>
<th>Solvent</th>
<th>TFC (mg/g) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>9.5±0.501</td>
</tr>
<tr>
<td>Methanol</td>
<td>14.3±1.13</td>
</tr>
<tr>
<td>Ethanol</td>
<td>12±0.595</td>
</tr>
<tr>
<td>Chloroform</td>
<td>7.8±0.655</td>
</tr>
<tr>
<td>Petroleum ether</td>
<td>4.21±1.09</td>
</tr>
</tbody>
</table>

Values are the average of triplicate experiments and values expressed as mean ± SEM.

and TFC is high which might be due to the different method of extraction and solvents polarities.

All leaf extract showed significant zone of inhibition against all the tested bacterial strains. Minimum zone of inhibition was observed against *M. smegmatis* (14 mm) and *B. subtilis* (15.7 mm) and Maximum was observed against the *E. coli* (19.2 mm) and *P. aeruginosa* (20.4 mm) Ethanolic extract showed highest zone of inhibition against *S. aureus* (17.3 mm) and *B. subtilis* (15.3 mm). The lowest zone of inhibition was recorded in *M. smegmatis* (13.6 mm) and *P. aeruginosa* (14.7 mm). Aqueous extract showed moderate activity only against *B. subtilis* (14.7 mm) and *S. aureus* (13.8 mm). Chloroform and petroleum ether showed activity only against *S. aureus* (13.4 mm) and *B. subtilis* (12.4 mm) respectively (Table 4, Figure 2). Among the different concentrations (50, 75 and 100 mg/mL of DMSO), 100 mg/mL produced maximum inhibitory activity against all the tested microorganisms. The results indicate that antibacterial activity of plants depends on the solvent used for extraction and tested bacterial strain. The inhibitory activity found directly dependent upon the concentration of plant extract used.

Goyal et al., (2008) worked the antimicrobial assay of *C. roseus* against the 6 different bacteria, reported that ethanolic extracts showed maximum inhibitory activities against *K. pneumonia* followed by *E. coli*. Sathiya et al., (2008) reported the methanolic extract of *C. roseus* to be more effective against the *B. subtilis*, *Klebsiella sp.*, *S. aureus* and *Streptococcus* sp. Balabirami and Patharajan, (2012) evaluated *C. roseus* against different bacterial and fungal strains with best results for antimicrobial activity with ethanolic extract. Govindasamy and Srinivasan, (2012) reported antimicrobial activities of *C. roseus* against 5 different bacterial strains with maximum inhibition zone was...
observed in ethanolic extract against the S. typhi followed by B. subtilis, S. aureus, P. aeruginosa and minimum against the E. coli. These phytochemical constituents previously reported with several biological activities such as antioxidant, antimicrobial, anti-fungal and anti-cancer (Hossain et al., 2014; Rashid et al., 2016). All these secondary metabolites showed antimicrobial properties through different mechanism. Saponins, cardiac glycosides and other type bioactive chemical constituents which are involved in plant disease resistance because of their antimicrobial activity. C. roseus plant has a very significant medicinal value.

The literature and publication in this area of research show that, C. roseus has mostly been studied with respect of anti-hypertension, anti-diabetic and anti-cancer properties (Balaabirami and Patharajan, 2012). Till now very few studies have been done on anti-bacterial properties. While no report were found related to M. smegmatis. Several authors already reported on flavonoids groups exhibited a wide range of biological activities such as antimicrobial, antioxidant, anti- analgesic, anti-inflammatory, anti-cancer and anti-allergic (Igbinosa et al., 2009). Moreover, many species of Apocynaceae family has been commonly used in traditional and folk medicine. Hence the present investigation on C. roseus plant extracts could be significant for the progress of new life preserving drugs. Further more advanced research is also required to isolate the new bioactive compounds.

CONCLUSION

It can be concluded from the above study that among the five solvents used for extraction, methanol and ethanol extract were found more affective, followed by aqueous. This may due to more solubility of bioactive compounds of C. roseus with methanol during the extraction process as compared to other solvents. Total phenolic and flavonoids content were found highest in methanolic extract. The extracts of leaf with different solvents were found to cause maximum antimicrobial activity toward B. subtilis followed by S. aureus and M. smegmatis. However, P. aeruginosa and E. coli was moderately sensitive against various solvents.

REFERENCES


