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In vitro anti-oxidant activity of aqueous and methanol extracts of *Catharanthus roseus* (L) G. Don

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Abstract
Catharanthus roseus is a well-known medicinal plant, a potential source of antioxidants, the compounds like polyphenols, flavonoids, and phenolic are present in the herbs; act as natural antioxidants which are responsible for inhibiting or preventing the consequence of oxidative stress by scavenging free radicals. In this study, antioxidant potential of aqueous and methanol extracts of *C. roseus* was determined by DPPH scavenging assay and Fe^{3+} reducing power assay. Both the extracts showed antioxidant activities at various concentrations. When compared to aqueous extracts methanol extracts shows highest amount of phenolic content, DPPH scavenging capacity and reducing capacity. The antioxidant potential of *C. roseus* extracts significantly correlated with the phenolic content of extracts. Ascorbic acid taken as control showed highest antioxidant power in the study.

Keywords: Antioxidant activity, *Catharanthus roseus*, DPPH scavenging and total phenolic content

Introduction
Catharanthus roseus is one of the renowned medicinal plants and an ornamental shrub which belongs to the family Apocynaceae. It was previously known as *Vinca rosea*, the plant originated from Madagascar Island and also known as Madagascar periwinkle. It is widely distributed around the world and has a greater capability to tolerate a wide range of climatic conditions, such as high ambient temperature, drought as well as heavy rainfall. *C. roseus* is widely cultivated in warmer regions of the world. It is a perennial, evergreen shrub with 30-100cm height. Commonly, there are two varieties of plant based on the flower colour; pink flowered *rosea* and white flowered *alba*. The leaves are glossy, dark green (1-2 inch long), oblong-elliptic, acute, rounded apex. It blooms throughout the year and propagated by seeds or cuttings (Van Bergen and Snoeijer, 1996) [51].

The plant has traditionally been used to treat wide varieties of diseases like menorrhagia, rheumatism, dyspepsia, indigestion, diabetes, hypertension, menstrual disorders, skin diseases and bleeding. Leaf extract has been used to treat wasp stings, cancer and cough. The areal part is taken orally as menstrual regulators and also used for relieving muscle pain as well as depression of the central nervous system. Moreover, some use *C. roseus* as a cold remedy to ease lung-congestion and inflammation. It is a solution to treat eye irritation and eye infections (Farnsworth., 1961; Swanston- Flat *et al*., 1989; Van Bergan, 1996) [10, 46, 51].

C. roseus also contains a significant number of volatiles and phenolic compounds. Phenolics comprise the principle secondary metabolites present in the plants, aims for the defence system against various biotic and abiotic stresses including anti-herbivore. *C. roseus* also contains a significant number of volatiles and phenolic compounds. Caffeoylquinic acids, flavonol glycosides, Rutin, Quercetin and Kaempferol are the most important phenolic compounds present in the *C. roseus* and with strong antioxidant activity. Phenolic compounds play an important role in plant driven anti-oxidant activity through adsorbing or neutralising the free radicals. Antioxidants in the cells act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chealaters or decomposing peroxides. All parts of *C. roseus* exhibit antioxidant properties (Djeridane *et al.*, 2006; Mustafa and Verpoorte, 2007; Ferreres *et al*., 2008; Pereira *et al.*, 2010; Ferreres *et al*., 2011) [8, 26, 12, 11].

Besides antioxidant and anti-cancerous activity, the plant compounds exhibit anti-diabetic, anti-allergic, anti-inflammatory, anti-microbial, anti-thrombotic, anti-helminthic, anti-ulcer, anti-diarrheal, wound healing, hypotensive, hypolipidemic, memory enhancement,

cardioprotective and vasodilator activities (Pillay *et al*., 1959; Chattopadhyay *et al*., 1991; Sekar, 1996; Nayak *et al*., 2007; Patil and Ghosh, 2010; Rajput *et al*., 2011; Patel *et al*., 2011) [36, 5, 42, 27, 32, 38, 31]. The secondary compounds of *C. roseus* are also incorporated into a wide range of commercial and industrial applications, served as resources for flavours, aromas and fragrances, bio-based fuels and plastics, enzymes, preservatives, cosmetics (cosmeceuticals), natural pigments, and bioactive compounds. The extracts of *C. roseus* act as a very strong inhibitor for mild steel corrosion (Shahba *et al*., 2016; Rana *et al*., 2017) [43, 39]. The phytochemicals present in the *C. roseus* contain a combination of many toxic compounds (Kneifel *et al.,* 2002; Thanaboripat *et al.,* 2007) [24, 49], which are beneficial at physiological doses, but high doses elicit adverse effects, thereby establishing a double-edged sword. The phytochemicals enter into the biological system and alter the metabolism leading to adverse outcomes. Because of these, toxicological studies have become mandatory to assess the safety of the compounds of phytochemical origin. Several studies have reported the toxic effects of the plant compounds in different experimental animals (Alaguchamy and Jayakumararaj, 2015) [1].

Materials and Methods

Extraction of phytochemicals

The fresh *Catharanthus roseus* plants were collected from Calicut University campus, Kerala, India and washed thoroughly in water. Shade dried samples were powdered using an electric grinder. The bioactive compounds were extracted from 50gm uniformly mixed dry powder in 750ml of methanol and water using Soxhlet apparatus with 500ml extraction cup. The extraction process was performed in 30 continuous cycles.

Methanol extraction

The dried powder homogeneously sampled in a pre-heated thimble and extracted in 750ml of crude methanol as solvent using Soxhlet apparatus at 64.7°C. After completion of 30 cycles, the extract was dried through evaporation using the rotary evaporator (Cyber lab, RE10 CSE84) under vacuum at 40°C. The dried extract was stored in airtight desiccators for further experiment.

Aqueous extraction

The dried powder of the *C. roseus* was taken in a pre-heated thimble filter and extraction process was carried out using Soxhlet apparatus with 750 ml of double distilled water at 100°C for 30 cycles. The extract was dried through evaporation and sample stored in an airtight desiccator.

Determination of total polyphenol content

The total polyphenols were measured using the Folin-Ciocalteu's spectrophotometric method by Box (1983). Polyphenols contain aromatic hydroxyl group that reacts with the Folins reagent to form a blue colour suitable for spectrophotometric estimation at a wavelength of 700nm.

Polyphenol estimation was conducted by taking different concentrations of standard (Gallic acid) and similarly in methanol extract and aqueous extract. The samples were made up to 5ml with deoxygenated water and 0.1ml of Folin phenol reagent was added in rapid succession. 1ml of carbonate tartrate reagents was added immediately (carbonate tartrate reagent was prepared by dissolving 100g Na_2CO_3 and 6g Sodium tartrate ($Na_2C_4H_4O_6.2H_2O$) in 375ml hot distilled water, cool to 20ºC and then diluted to 500ml). The reaction tubes in duplicate were kept at room temperature for 30 minutes. After incubation, the absorbance was measured at 700nm using a spectrophotometer. Total phenolic content was calculated from the calibration curve of Gallic acid and the result was expressed as milligram of Gallic acid equivalent per gram dry weight.

Reducing power activity

Reducing power activity of a compound indicates its antioxidant activity. The Fe^{3+} reducing power of the extract was determined by the method of Oyaizu (1986) [29]. The aqueous extract (mg/L) and methanol extract (mg/L) at various concentrations were mixed with 2.5ml of phosphate buffer (pH 6.6) and 2.5ml of potassium hexacyanoferrate [$K_3Fe(CN)_6$] (1%, w/v), followed by incubating at 50ºC in a water bath for 20min. The reaction was stopped by adding 2.5ml of trichloroacetic acid (TCA) solution (10%) and then centrifuged at 3000 rpm for 10 minutes. 2.5ml of the supernatant was mixed with 2.5ml of distilled water and 0.5ml of ferric chloride ($FeCl_3$) solution (0.1%, w/v) for 10 minutes. The presence of reducers (antioxidants) causes the reduction of the Fe3+/ferrous cyanide complex to the ferrous form and measuring the formation of Perl's Prussian blue by absorbance at 700nm. The absorbance was measured in the spectrophotometric instrument (UV-VIS 1800, Spectrophotometer, Shimadzu, Japan) and monitors the Fe2+ concentration. The higher absorbance of the reaction mixture indicated greater reducing power. The assays were carried out in triplicate and the results are expressed as mean values ± standard deviations. The extract concentration providing 0.5 of absorbance was calculated from the graph of absorbance at 700 nm against extract concentration. Gallic acid was used as standards.

Free radical scavenging activity with DPPH assay

The free radicals scavenging activities of *C. roseus* were assayed using a stable DPPH, following standard method (Blois, 1958) with slight modification. DPPH is a common abbreviation for an organic chemical compound 2, 2-diphenyl-1-picrylhydrazyl (also known as 1,1-diphenyl-2-picrylhydrazyl radicals, 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl, diphenyl picryl-hydrazyl). It is a dark coloured crystalline powder composed of stable free radical molecules. For the present study, DPPH was procured from Sigma Aldrich. 5mg DPPH powder weighed in the semi-analytical electronic balance (Shimadzu -Ax200, Japan) and makeup to 25ml by using methanol and kept in the dark so as to avoid photo bleaching. The reaction mixture contains 200µl of 100µM DPPH solution and 50µl different concentration of (0.5µl, 1µl, 2µl, 5µl, 10µl) methanol (28.4µg/µl) and water (27.5µg/µl) extract of *C. roseus*. The mixture was then incubated in dark for 30 minutes at 37ºC and the absorbance at 517nm was recorded as the sample (A_{Sample}), using 96 well microplate reader (SYNERGY HT, USA).

A blank experiment was also carried out, applying the same procedure to a solution and the absorbance was recorded as blank (A_{Blank}). The free radicals scavenging activity of each solution was then calculated as percent inhibition according to the following equation.

$$\% \text{ of inhibition} = 100 \times (A_{Blank} - A_{Sample})/A_{Blank}$$

Statistical analysis

The results were processed through computer programs: Excel and SPSS software (version 20). Student's t-test was

used to determine significant differences in polyphenols and antioxidant activity in methanol and aqueous extract. Correlation coefficient test was applied to find the association between antioxidant components and antioxidant activity of both extracts. Probability level was fixed to $P < 0.05$. The data were expressed as means ± SD.

Result and Discussion

Total polyphenol estimation

Total polyphenolic content in aqueous and methanol extract of *C. roseus* was calculated from the calibration curve (R^2 = 0.9998) of gallic acid and expressed the result in mg of gallic acid equivalent. Table.1 shows the total polyphenol concentration in the extracts. The total phenolic content in aqueous and methanol extract were 32.07±0.3032mgGAE/g and 35.96 ± 0.1815mg GAE/g respectively

Table 1: Total polyphenol content of aqueous and methanol extracts (mg GAE/gram)

Extracts	Total phenolic content (mg GAE/g) mean ±SD, n=6
Aqueous extract	32.07 ± 0.3032
Methanol extract	35.96 ± 0.1815

Reducing power assay

Ascorbic acid used as standard shows the highest antioxidant activity (Figure.1). The reducing power of aqueous and methanol extract of *C. roseus* were analysed and presented in the Figure. 2. There is a strong positive significant correlation between reducing power and concentration in ascorbic, methanol extract and aqueous extract with correlation coefficient 0.970, 0.963 and 0.963 respectively, and the $P<0.05$.

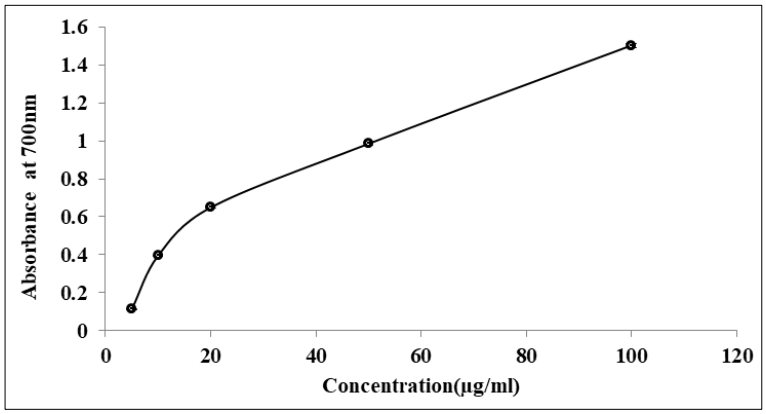

Fig 1: Reducing power of ascorbic acid

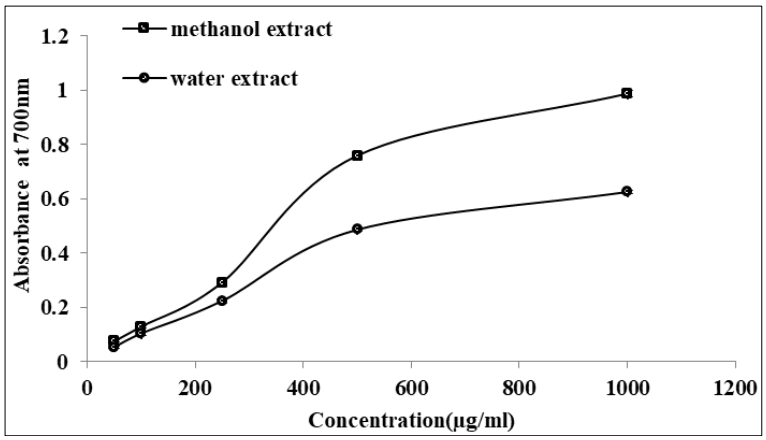

Fig 2: Reducing power of aqueous and methanol extracts of *C. roseus*

Free radical scavenging activity with DPPH assay

The radical scavenging activity of methanol and aqueous extracts from *C. roseus* were examined and the results are expressed as the ratio percentage of sample absorbance of extract at 517 nm. There was a strong positive correlation between scavenging activity against concentration in methanol and aqueous extract with correlation coefficient of 0.816 and 0.813 respectively (Figure.3). Scavenging property expressed as IC_{50} value (Figure.4). IC_{50} value was determined from the plotted graph of scavenging activity against various concentrations of aqueous and methanol extract. The activity is defined as the effective concentrations of antioxidant necessary to decrease the initial DPPH radical concentration by fifty percentages. The lowest IC_{50} indicates the strongest ability to extract to act as radical scavengers. Out of two extracts, methanol extract showed lowest IC_{50} (128.3µg/ml) whereas the aqueous extract showed IC_{50} of 416.74µg/ml. The IC_{50} value of ascorbic acid was 41.135µg/ml, showed higher radical scavenging activity (Figure 5).

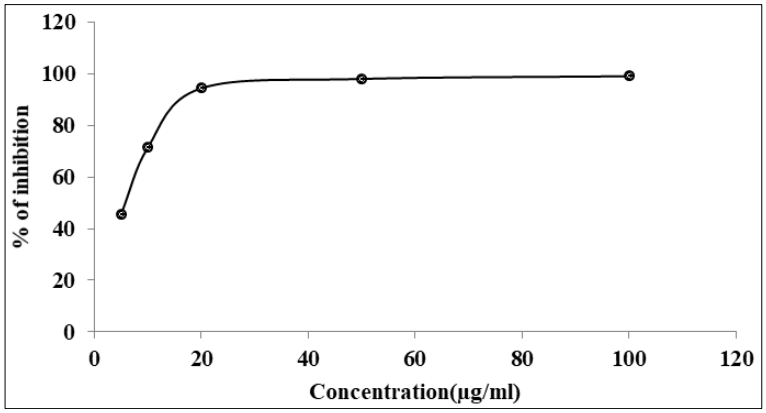

Fig 3: DPPH scavenging activity ascorbic acid

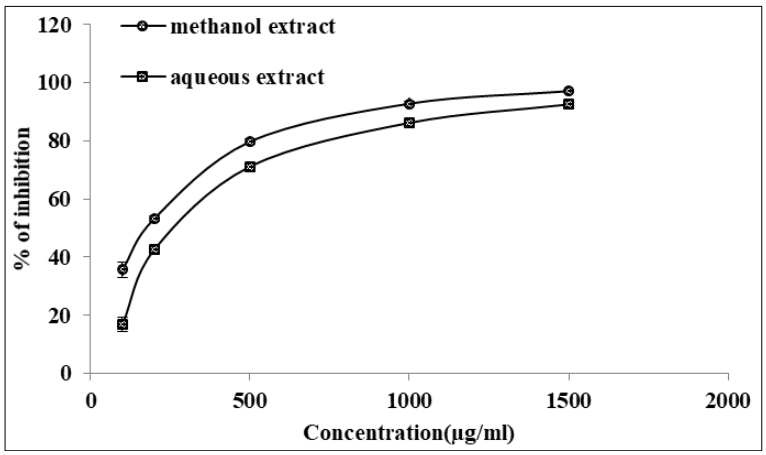

Fig 4: DPPH scavenging activity extract aqueous and methanol extracts

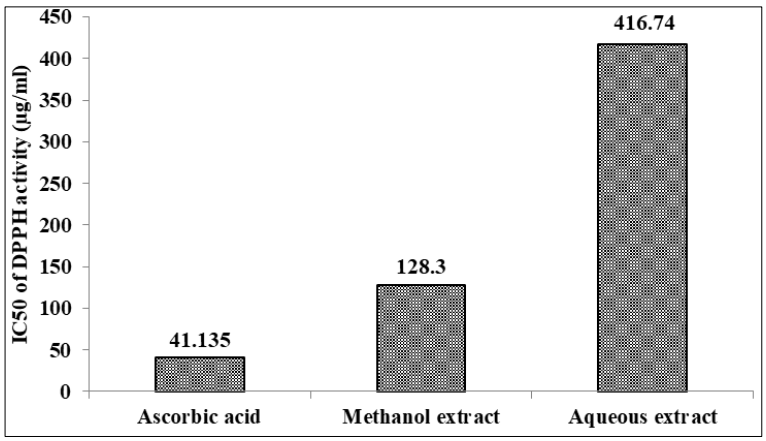

Fig 5: IC_{50} of DPPH scavenging activity of ascorbic acid, aqueous extract and methanol extract

Discussion

Plants widely used in various traditional medicines because of their antioxidant properties and pharmacological actions. The secondary metabolites present in the plants act as natural antioxidants which are responsible for inhibiting or preventing the consequences of oxidative stress by scavenging free radicals. *Catharanthus roseus* is an important medicinal plant and it is a potential source of antioxidant activity. It is a principle ingredient in many Ayurvedic formulations (Ferreres *et al*., 2008) [12]. This study analysed the antioxidant activity of aqueous and methanol extracts of *C. roseus* along with their total phenolic content.

Polyphenol compounds are important constituents of plant secondary metabolite with unique properties of their free radical scavenging ability due to hydroxyl groups. The different phenolic anti-oxidants such as flavonoids, tannins, coumarins, xanthine etc. are scavenging the free radicals dose-dependently. Thus, they have been used as therapeutic drugs against free radical pathologies (Gutteridge, 1995; Kahkonen *et al*., 1999; Hutchon and El-Gabalawy, 2004; Geronikaki and Gavalas, 2006) [14, 21, 16, 13]. Present result shows that the total polyphenolic content of methanol extracts is slightly higher than aqueous extracts (Table.6).

The anti-oxidant activity of botanicals is correlated with the abundance of phenolic compounds (Velioglu *et al*., 1998) [52]. Cheruparambath *et al*. (2015) [6] reported that the methanol extract had the highest polyphenol content compared to aqueous extracts of Dahlia flower. Kumar *et al*. (2012) [25] reported that methanolic leaf extract of *C. roseus* has a rich source of phenolic compounds. The present study is also supported by the results of Kabesh *et al*. (2015) [20]. The total polyphenol content of methanol extract contains a high

amount of polyphenols than the aqueous extract of *C. roseus* through Folins Ciocalteu method. Rasool *et al.* (2011) [40] also confirmed the result, 100% methanolic extract of *C. roseus* showed the highest polyphenol content (19.8g/ 100g).

The antioxidant activity of different parts of *C. roseus* has been demonstrated in earlier studies (Pereira *et al*., 2010; Rasool *et al*., 2011; Kumar *et al*., 2012; Widowati *et al*., 2013) [33, 40, 25, 53]. The anti-oxidant ability of methanol and aqueous extract of *C. roseus* (whole plant) was studied and results are included in (Figure.4-8). The reducing power of plant extract was a reflection of the anti-oxidant activity (Oktay *et al*., 2003) [28]. Reducing power assay is used to evaluate the antioxidant ability of a substance to donate an electron (Yildirim *et al*., 2000) [54]. Antioxidants are reductones and they act as electron donors and reacts with free radicals to convert them into more stable products and terminate the free radical chain reactions. Irshad *et al*. (2012) [17] reported that methanol extract of *Cassia fistula* has the highest reducing power compared to hexane extract. The antioxidant activity of the extracts was strongly dependent on the extraction solvent (Jang *et al*., 2007) [19].

DPPH free radical scavenging assay is widely used to assess the antioxidant activity of plant extracts (Philips *et al*., 2010) [35]. This method is based on the reduction of the free radical in the presence of hydrogen-donating antioxidant due to their capability to accept an electron or hydrogen radical from DPPH. The remaining DPPH measured after 30 minutes, corresponds inversely to the radical scavenging activity of the antioxidant decreasing its absorbance value at 517 nm. In the present study, the DPPH scavenging activity of methanol and aqueous extract of *C. roseus* were increasing with concentrations and methanol extracts showed the higher scavenging activity when compared to aqueous extract (Figure.7). The antioxidant activity of the methanol and aqueous extracts were compared to their total phenolic content. The radical scavenging activities of the extracts directly proportional to the phenolic content present in the extracts.

The present results showed a greater rate of DPPH scavenging activity in methanol extracts when compared to the aqueous extracts. The effective concentration of antioxidant required to decrease the initial DPPH radical concentration by 50% (IC_{50}) was interpolated from the plotted graph of scavenging activity against various concentrations of the extracts (Figure. 8). The lowest IC_{50} value indicates the strongest ability of the extracts to act as free radical scavengers. Out of two extracts, methanol extract showed the lowest IC_{50} (128.3µg/ml), whereas aqueous extracts showed the highest (416.74µg/ml). Sun and Ho (2005) [45] reported that a significant correlation exists between total phenolic components and scavenging ability of buckwheat (*Fagopyrum esculentum*) extracts on DPPH radicals. And Irshad *et al*. (2012) [17] also reported that the antioxidant ability extract of *Cassia fistula* was directly proportional to phenolic content. Several studies reported the positive correlation between the total phenolic content and the antioxidant activities of the plants (Kim *et al*., 2003; Djeridane *et al*., 2006; Bouayed *et al*., 2007; Lim & Quah, 2007; Tawaha *et al*., 2007) [23, 8, 2, 3, 9]. This study clearly indicated that the methanol extracts of *Catharanthus roseus* exhibited high content of phenolic compounds which was significantly correlated with the DPPH radical scavenging activity. The aqueous and methanol extracts of *Catharanthus roseus* have antioxidant properties. The compounds present in the plant act as exogenous antioxidants and exhibit beneficial effects, in order to prevent oxidative stress in the organisms. The balance between oxidation and antioxidation is maintained in the biological systems. High doses of exogenous antioxidants may disrupt redox balance (double edged-effects in the cellular redox state) and produce harmful effects. Hence physiological doses are required to maintain redox homeostasis (Ratnam *et al*., 2006; Valko *et al*., 2007; Bouayed *et al*., 2009) [41, 50, 4]. The present study demonstrated the dose-dependent eclosion inhibition for calculating the physiological doses of the aqueous and methanol extracts of *C. roseus.*

The harmful effects of *C. roseus* (insecticidal properties) have been reported in earlier studies. The study of Deshmukhe *et al*. (2010) [7] showed that the crude aqueous leaf extract of *C. roseus* has insecticidal potential against *Spodoptera litura fabricius* and the report of Prajapati *et al*. (1998) [37] concluded that the root extracts of *C. roseus* exhibit anti-feedent and growth inhibiting activities. The eclosion inhibition may be due to extract's repellence and toxic effect since they contain phenolics and alkaloid constituents with pesticidal property (Hassan *et al*., 2011) [15]. The petroleum ether extract of the same plant was used to control *Anopheles stephensi* mosquitos (Panneerselvam *et al*., 2013) [30]. Vincristine and vinblastine, important anticancer alkaloids present in the *C. roseus*, which cause neurotoxicity and influence motor and neuronal function along with bone marrow depression in human (Tayeb *et al*., 2010) [48].

The present result shows that the rate of eclosion inhibition is higher in methanol extract when compared to aqueous extract. The efficiency of the phenolic extraction depends on the type of the plant and the kind of solvent used (Jakopic and Veberic, 2009). Kevin *et al*. (2012) [22] also reported that methanol leaves extract of *C. roseus* caused diarrhoea and mortality in the female rat. The magnitude of extraction yield of *Hieracium pilosella* leaf was higher in methanol when compared to water (Stanojevic *et al*., 2009) [44]. Hence the efficacy of methanolic extraction increases due to its higher yield.

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Conflict of Interest

No conflict of interest its associated with this work.

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