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In vitro Antioxidant Activity, Phytochemical Screening, Cytotoxicity and Total Phenolic Content in Extracts of *Caesalpinia pulcherrima* (Caesalpiniaceae) Pods

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Abstract: *Caesalpinia pulcherrima* L. Swartz (Caesalpiniaceae) is an ornamental plant also used as a common medicinal plant in India, Taiwan and South-East Asian countries. Majority of the diseases/disorders are mainly linked to oxidative stress due to free radicals. The aims of this study were to screen for phytochemical constituents, evaluate cytotoxicity, *in vitro* antioxidant activity and estimation of total phenolic content of extracts of pods of *Caesalpinia pulcherrima*. Phytochemical analysis revealed the presence of tannins, flavonoids, steroids and alkaloids. Brine Shrimp Lethality (BSL) bioassay was used to investigate the cytotoxic effects. The LC_{50} ($\mu g\ mL^{-1}$) values obtained for extracts as 750 $\mu g\ mL^{-1}$ for petroleum ether extract, 800 $\mu g\ mL^{-1}$ for chloroform extract and 900 $\mu g\ mL^{-1}$ for methanol extract. The total phenolic content of the methanolic extract was 38.04% w/w, equivalent to gallic acid. Petroleum ether, chloroform and methanolic extracts of *Caesalpinia pulcherrima* and standard ascorbic acid were found to be scavenger of DPPH radical with an IC_{50} of 124.75, 112.08, 54.34 and 13.86 $\mu g\ mL^{-1}$, respectively. Methanolic extract was good scavenger of DPPH radical. Petroleum ether, chloroform, ethyl acetate soluble fraction of methanolic extracts of pods of *Caesalpinia pulcherrima* and ascorbic acid were found to be scavenger of nitric oxide radical with an IC_{50} of 93.32, 65.12, 54.83 and 12.59 $\mu g\ mL^{-1}$, respectively. Ethyl acetate soluble fraction was found to be good scavenger of nitric oxide radical. Our conclusion provides support that the crude extracts of *C. pulcherrima* is a probable source of natural antioxidants and this justified its uses in folkloric medicines.

Key words: *Caesalpinia pulcherrima*, cytotoxicity, brine shrimp lethality test, total phenolic content, antioxidants

INTRODUCTION

In Ayurveda, the use of herbal extracts and nutritional supplements is well documented either as alternative or complimentary medicine to the conventional chemotherapy for treatment of inflammatory diseases in the Indian subcontinent for 5000 years (Dahanukar *et al*., 2000). The current rising recognition of medicinal plants is due to many reasons, including escalating faith in herbal medicine. No doubt allopathic medicine may cure a wide range of diseases; however, its high prices and side-effects are causing many people to return to herbal medicines which have fewer side effects (Kala, 2005). For most of the developing world, the main issue of public health is still the acute need for basic health care which is sadly lacking even at the most elementary level. This is true in both the rapidly growing cities and in the rural areas. The World Health Organization (WHO) indicates that more than half of the world's population does not have access to adequate health care services. Medicinal plants offer alternative remedies with tremendous opportunities. They not only provide access and affordable medicine to poor people; they can also generate income, employment and foreign exchange for developing countries. Many traditional healing herbs and plant parts have been shown to have medicinal value, especially in the rural areas and that these can be used to prevent, alleviate or cure several human diseases. The WHO estimates that more than 80% of the world's population rely either solely or largely on traditional remedies for health care. Interest in the exploitation of medicinal and aromatic plants as pharmaceuticals, herbal remedies, flavorings, perfumes and cosmetics and other natural products has greatly increased in the recent years (CIMAP, 2000). *Caesalpinia pulcherrima* L. (Caesalpiniaceae) is an attractive plant due to its array of flowers which appear yellow, pink, off-white and red with yellow margins (Roach *et al*., 2003). *Caesalpinia*

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pulcherrima is also known as peacock flower. It is a common medicinal plant in India, Taiwan and South-East Asian countries. In alternative medicine, the different parts of this plant have been used as an anti-inflammatory, abortifacient, emmenagogue, bronchitis and malarial infection while fruits are employed to cure diarrhea and dysentery (Srinivas *et al*., 2003; Chiang *et al*., 2003). Bark shown Antimicrobial, Cytotoxic activity (Islam *et al*., 2003). Flowers shown Antimicrobial and Antifungal activity (Sudhakar *et al*., 2006), fruits shown Antiviral activity (Chiang *et al*., 2003). Leaves shown Antitumor activity (Chiang *et al*., 2003), Antimicrobial activity (Ragasa *et al*., 2002), Antiviral activity (Chiang *et al*., 2003). Its seeds have shown Antiviral activity (Chiang *et al*., 2003) stem shown cytotoxic activity (McPherson *et al*., 1983). The brine shrimp cytotoxicity assay was considered as a convenient probe for preliminary assessment of toxicity developed by Meyer *et al*. (1982). Since ancient times, the medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities. As antioxidants have been reported to prevent oxidative damage caused by free radicals, it can interfere with the oxidation process by reacting with free radicals, chelating, catalytic metals and also by acting as oxygen scavengers (Shahidi *et al*., 1992; Elzaawely and Tawata, 2012). Akond *et al*. (2011) reported total polyphenol and antioxidant activity of 29 common bean from diverse origins USA, Barzil and India. The potentially reactive derivatives of oxygen, attributed as Reactive Oxygen Species (ROS), are continuously generated inside the human body. ROS have been considered to cause injury to living organisms and thus play an important role in many human diseases such as arthritis, atherosclerosis, diabetes mellitus and cancer (Elzaawely and Tawata, 2012; Gupta *et al*., 2007). The generated ROS are detoxified by the antioxidants present in the body. Recently there has been an increase of attention in the therapeutic potentials of medicinal plants as antioxidants in reducing such free radical induced tissue injury. Besides well identified and traditionally used natural antioxidants from tea, wine, fruits, vegetables and spices, some natural antioxidant (e.g., rosemary and sage) are already exploited commercially also as antioxidant additives or nutritional supplements (Schuler, 1990). Also many other plant species have been investigated in the search for novel antioxidants (Chu *et al*., 2000; Koleva *et al*., 2002; Mantle *et al*., 2000; Oke and Hamburger, 2002) but generally there is still a demand to find more information concerning the antioxidant potential of plant species. It has been mentioned the antioxidant activity of plants might be due to their phenolic compounds (Cook and Samman, 1996). Flavonoids are a group of polyphenolic compounds with known properties which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action (Frankel, 1995). Some evidence suggests that the biological actions of these compounds are related to their antioxidant activity (Gryglewski *et al*., 1987). An easy, rapid and sensitive method for the antioxidant screening of plant extracts is free radical scavenging assay using 1,1-diphenyl-2-picrylhydrazyl (DPPH) stable radical spectrophotometrically. In the presence of an antioxidant, DPPH radical obtains one more electron and the absorbance decreases (Koleva *et al*., 2002). Phenols are a class of low molecular weight secondary metabolites found in most land plants (Okpuzor *et al*., 2009). Derived polyphenols from plants are of great importance because of their potential antioxidant and antimicrobial properties. Phenolic compounds exhibit a considerable free radical scavenging (antioxidant) activity which is determined by their reactivity as hydrogen or electron-donating agents, the stability of the resulting antioxidant derived radical, their reactivity with other antioxidants and finally their metal chelating properties (Ang-Lee *et al*., 2001; Wojdylo *et al*., 2007). Phytochemicals have been of huge interest as a supply of natural antioxidants used for health promotion, food preservation, food flavoring and cosmetics as they are safer than synthetics (Khasawneh *et al*., 2011). In this study for the first time evaluated cytotoxicity, *in vitro* antioxidant activity and estimated total phenolic content of methanolic extract of pods of *Caesalpinia pulcherrima* (Caesalpiniaceae).

MATERIALS AND METHODS

Plant material: Pods of *Caesalpinia* pulcherrima was collected from local region of Nashik, India in October 2008. The plant material was identified and authenticated by Dr. P.G. Diwakar Botanical survey of India, Koregaon Park, Pune, India. (Ref No. BSI/WC/Tech/2009/370).

Preparation of extract: The plant material were cleaned, dried under shade and pulverized by using grinder. 500 g of the powder of pods was in succession extracted with petroleum ether, chloroform and methanol in order of their rising polarity using Soxhlet apparatus. The yield of extracts obtained as petroleum ether as 1.21%, chloroform as 2.46%, Methanol as 13.32%. From the Preliminary Phytochemical study revealed that occurrence of sterols, glycosides, alkaloids, triterpenoids, flavonoids and tannins in the extracts.

Brine shrimp lethality (BST): The *in vitro* lethality in a simple zoological organism such as BST, developed by Meyer *et al.* (1982), might be used as a simple tool to guide for cytotoxic activity. Brine shrimp eggs were collected from Fisheries Dept. Government of Maharashtra Dapachari, Dahanu Dist-Thane, Maharashtra, India. Brine shrimp eggs were placed in artificial sea water (3.8% w/v NaCl in distilled water) and incubated at 24-28°C. Eggs were hatched in 48 h providing large number of larvae (nauplii). Ten numbers of nauplii were placed in 5 mL of sea water and different concentrations were prepared and placed in vials. Alive nauplii were counted after 24 h and lethal concentration (LC_{50}) calculated (Ramachandran *et al.*, 2011).

Antioxidant activity

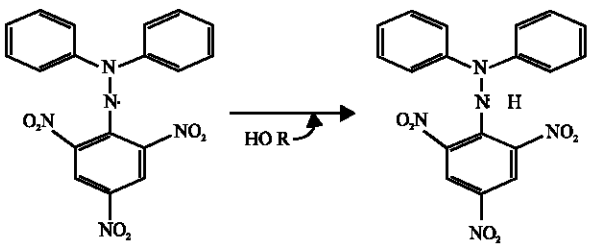
DPPH free radical scavenging assay: 2,2-diphenyl-1-picrylhydrazyl (DPPH) is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and to evaluate antioxidant activity of phytoconstituents. DPPH is nitrogen centered free radical. It reacts similar as peroxyl radial. The reaction rates directly correlate with antioxidant activity. The odd electron in DPPH free radical gives a strong absorption maximum at 517 nm and is purple in color. The color turn from purple to yellow as the molar absorptivity of DPPH radical at 517 nm reduces when odd electron of DPPH radical becomes paired with hydrogen from free radical scavenging antioxidant to form the reduced DPPH-H. The resulting decoloration is stoichiometric with respect to number of electrons captured (Sanchez-Moreno *et al.*, 1999).

Ascorbic acid was used as standard. Percentage inhibition was calculated using formula:

$$\text{Inhibition (\%)} = \frac{A_{blank} - A_{test}}{A_{blank}} \times 100$$

where, A is absorbance.

The respective reaction is as follows:



Nitric oxide radical scavenging assay: Sodium nitroprusside in aqueous solution at physiological pH, spontaneously produce nitric oxide which reacts with oxygen to produce nitrite ions which can be determined by the use of the Griess reagent. Scavengers of nitric oxide compete with oxygen and reduce the production of nitric oxide. Nitric oxide scavenging activity was performed by sodium nitroprusside-Griess reagent. In this method sodium nitroprusside (1 mM) in phosphate buffer saline solution was mixed with different concentration of extracts solution in methanol and incubated at 37°C for 150 min. Blank solution was also prepared. After incubation 0.5 mL of Griess reagent (1% sulphanilamide, 2% o-phosphoric acid add 0.1% N-(1-naphthyl)-ethylenediamine hydrochloride) was added. The absorbance was taken at 546 nm. Ascorbic acid was used as a standard. Percentage inhibition was calculated as per above formula. IC_{50} was calculated for each extract (Viturro *et al.*, 1999; Garrat, 1964).

Estimation of total phenolic content: Total phenolic content of methanol extract of pods of *Caesalpinia pulcherrima* by Folin-Ciocalteu method was determined as per method described by Chandler and Dodds (1993). The quantitative phenolic estimation was performed at max 765 nm by change in intensity of Folin-phenolic compounds complex. Readings were taken after 1 h at 765 nm by UV Spectrophotometer (1650 Shimadzu, Japan) against reagent blank. With the help of calibration curve, the phenolic concentration of extract was determined (Ravishankara *et al.*, 2002; Chandler and Dodds, 1993; Vicente *et al.*, 2011).

RESULTS

Phytochemical screening: The crude petroleum ether, chloroform and methanolic extract of pods of *Caesalpinia pulcherrima* was qualitatively tested for the presence of sterols, glycosides, alkaloids, triterpenoids, flavonoids, anthraquinones, carotenoids, tannins and the results were given in Table 1.

Cytotoxicity studies: In brine shrimp lethality bioassay (Table 2), crude petroleum ether, chloroform and methanolic extract of pods of *Caesalpinia pulcherrima* showed lethality against the brine shrimp nauplii. It showed different mortality rate at different concentrations. The LC_{50} ($\mu g\ mL^{-1}$) values obtained for extracts as 750 $\mu g\ mL^{-1}$ for petroleum ether extract, 800 $\mu g\ mL^{-1}$ for chloroform extract and 900 $\mu g\ mL^{-1}$ for methanol extract.

Antioxidant activity by DPPH free radical scavenging assay: DPPH radical scavenging ability is widely used as an index to evaluate the antioxidant potential of medicinal

Table 1: Preliminary phytochemical study

Test name	Detecting compound	Extracts of *Caesalpinia pulcherrima* pods		
		Petroleum ether	Chloroform	Methanol
Salkowaski test and Liberman test	Sterols	+	-	-
β-naphthol test	Glycosides	-	-	+
Dragendorff test, Mayer's test, Wagner's test and Hager's test	Alkaloids	-	-	-
Libermann-Burchard test	Triterpenoids	+	+	-
Shinodha test, antimony trichloride test and ferric chloride test	Flavonoids	-	-	+
Juglone test	Anthraquinones	-	-	-
Ferric chloride test and Matchstick test	Carotenoids	-	-	-
Carr-Price test	Tannins	-	-	+

+: Positive test, -: Negative test

Table 2: LC_{50} values of different extracts of *Caesalpinia pulcherrima* pods

Extract	LC_{50} (μg mL^{-1})
Petroleum ether	750
Chloroform	800
Methanol	900

Table 3: DPPH free radical scavenging activity of different extracts of *Caesalpinia pulcherrima* pods and ascorbic acid

Test component	Conc. (μg mL^{-1})	Inhibition (%)	IC_{50} (μg mL^{-1})
Petroleum ether extract	25	16.80	144.58
	50	23.20	
	75	28.46	
	100	36.12	
Chloroform extract	25	11.26	102.88
	50	21.13	
	75	33.56	
	100	52.58	
Methanol extract	25	21.50	64.519
	50	39.10	
	75	59.07	
	100	76.11	
Ascorbic acid	5	15.64	13.86
	10	34.51	
	15	51.45	
	20	73.87	
	25	91.93	

plants. *In vitro* antioxidant studies of the three extracts, the extent of DPPH radical scavenging at different concentrations (25-100 μg mL^{-1}) of *Caesalpinia pulcherrima* extracts was measured, with ascorbic acid as the standard/control. The radical scavenging effect was found to increase with increasing concentrations. The control and the plant extracts showed (Table 3) their maximum activity of 91.93% (control) 76.11% (methanol) 52.58% (chloroform) 36.12% (petroleum ether), respectively with IC_{50} values of 13.86, 64.51, 102.88 and 144.58 μg mL^{-1}. Methanolic extract was found to be good scavenger of DPPH radical.

Antioxidant activity by nitrous oxide free radical scavenging assay: Nitric oxide radical scavenging activity was determined according to the method reported by Garrat (1964). *In vitro* antioxidant studies of the three extracts; the extent of NO radical scavenging at different concentrations (25-100 μg mL^{-1}) of *Caesalpinia pulcherrima* extracts was measured, with ascorbic acid as the standard. The radical scavenging effect was found to increase with increasing concentrations. The control and the plant extracts showed (Table 4) their maximum activity of 93.78% (control) 70.40% (methanol) 66.67% (chloroform) 46.13% (petroleum ether), respectively with IC_{50} values of 12.59, 68.44, 73.08 and 107.59 μg mL^{-1}.

Table 4: Nitric oxide free radical scavenging activity of different extracts of *Caesalpinia pulcherrima* pods and ascorbic acid

Test component	Conc. (μg mL^{-1})	Inhibition (%)	IC_{50} (μg mL^{-1})
Petroleum ether extract	25	13.26	107.59
	50	22.29	
	75	36.38	
	100	46.13	
Chloroform extract	25	19.13	73.08
	50	34.39	
	75	52.84	
	100	66.67	
Methanol extract	25	16.73	68.44
	50	39.13	
	75	57.09	
	100	70.40	
Ascorbic acid	5	25.17	12.59
	10	48.29	
	15	63.18	
	20	78.19	
	25	93.78	

Table 5: Calibration curve data of gallic acid

Concentration (μg mL^{-1})	Absorbance
01	0.374
02	0.498
03	0.567
04	0.699
05	0.791
06	0.904
07	1.005
08	1.147
Methanolic extract of *Caesalpinia pulcherrima*	1.354

Estimation of total phenolic content: The values obtained for absorbance at 765 nm were 0.374, 0.498, 0.567, 0.699, 0.791, 0.904, 1.005, 1.147 and 1.354, respectively for 1, 2, 3, 4, 5, 6, 7, 8 and methanolic extract of *Caesalpinia pulcherrima*. Total phenolics content in methanolic extract of pods of *Caesalpinia pulcherrima* was found to 38.04% w/w, equivalent to gallic acid. Result is shown in Table 5.

DISCUSSION

The crude petroleum ether, chloroform and methanolic extracts of pods of *Caesalpinia pulcherrima* was qualitatively tested for the presence of sterols, glycosides, alkaloids, triterpenoids, flavonoids, anthraquinones, carotenoids and tannins. Isolation of pure, pharmacologically active constituents from plants remains an extensive and tiresome process. For this reason, it is necessary to have methods existing which eliminate needless separation procedures. Chemical screening is thus performed to allow localization and targeted isolation of new or valuable constituents with possible pharmacological activities. Alkaloids have been linked with medicinal uses for centuries and one of their common biological properties is their cytotoxicity (Nobori *et al*., 1994). The selection of today's therapy is therefore investigation of plant drugs. However, due to the over exploitation of the medicinal plants, several of them have become rare. The brine shrimp lethality assay represents a quick, inexpensive and simple bioassay for testing plant extracts bioactivity which in the majority cases correlates reasonably fit with cytotoxicity and anti-tumor properties. Cytotoxic and antioxidant activity displayed by the plants demonstrates presence of secondary metabolites (McLaughlin *et al*., 1993). In this study, the brine shrimp lethality of extracts of pods of *Caesalpinia pulcherrima* a medicinal plant used in traditional medicine to brine shrimp was determined following the modified method of Solis *et al*. (1993). Cytotoxic property by plant material is due to the presence of antitumor compounds (Ara *et al*., 1999). Cancer is the main killer disease in most developed as well as developing countries which is induced by oxidative stress (Bandyopadhyay *et al*., 1999; Gulcin, 2009). Hence antioxidants which are very effective in combating cancer needs thorough search especially safer compounds from plant sources. Increased oxidative stress encountered in body due to either environmental hazard or impairment in the body metabolism due to varying disease conditions including drugs or having insufficient amount of dietary antioxidants, has to be limited by exogenous supply of antioxidants as a choice of therapy or preventive measure. Natural antioxidants that are present in herbs and spices are accountable for inhibiting or preventing the harmful consequences of oxidative stress. Spices and herbs contain free radical scavengers like polyphenols, flavonoids and phenolic compounds Natural antioxidants are favored over that of allopathic drugs to overcome the side effects. The majority of the polar compounds such as phenolic and flavonoid substances are potent inhibitors of reactive oxygen species attack (Owen *et al*., 2003; Rebiai *et al*., 2011). Phenol compounds have some antioxidant activity (Leamsomrong *et al*., 2009). They are able to terminate free radicals and chelate metal ions that are capable of catalyzing formation of ROS that promote lipid peroxidation (Muchuweti *et al*., 2007). There are many studies indicated on the toxic effects of plants attributed to the presence of bound cyanogenic glycosides (Zakaria *et al*., 2006). The biological properties, including cytotoxic and antioxidant properties, of flavonoids are considered in an evaluation of the medicinal and nutritional values of these compounds (Harborne and Williams, 2000; Ramachandran *et al*., 2011). The antioxidant activity has correlation with total phenolic content. Methanolic, chloroform and petroleum ether extracts at various concentrations ranging from 25-100 μg mL^{-1} were tested for their antioxidant activity using DPPH (1-diphenyl-2-picrylhydrazyl) radical scavenging assay method. The DPPH is a stable free radical at room temperature and accept an electron or hydrogen radical to become a stable diagnostic molecule (Gupta and Sharma, 2010). This study explains antioxidant activity of extracts. DPPH radical scavenging activity assay assesses the capacity of the extract to donate hydrogen or to scavenge free radicals. The result revealed that the methanolic extract of the species exhibited the highest radical scavenging activity (%) with 76.11 followed by its chloroform extract with 52.58 and for petroleum ether extract 36.12. The DPPH radical scavenging activities of extracts increased gradually in a dose dependent manner. Smaller IC_{50} value corresponds to a higher antioxidant activity of the plant extract (Maisuthisakul *et al*., 2007). Nitric oxide is a very unstable species under the aerobic condition. It reacts with O_2 to produce the stable product nitrates and nitrite through intermediates through NO_2, N_2O_4 and N_3O_4. It is estimated by using the Griess reagent. In the presence of test compound which is a scavenger, the amount of nitrous acid will decrease. Free radicals are constantly produced in the living system which can cause an extensive damage to bio-molecules and tissues thereby causing various diseases like extensive lysis and degenerative diseases (Ames *et al*., 1993). The results obtained are shown in Table 4 and it indicates that the crude methanolic extract (70.4%), chloroform extract (66.67%) and petroleum ether extract (46.13%) of the plant possessed moderate NO free radical scavenging activity. The NO free radical scavenging activity was increased by increasing the concentration of the test samples. Plant phenolics are a major group of compounds acting as primary antioxidants or free radical scavengers. Therefore, it was reasonable to determine the total phenolic content in the plant extract. The result shows that the phenolic content of pods of

Caesalpinia pulcherrima is higher and the radical scavenging activity is likely to be due to the phenolics however, phenols may not be solely responsible for the antioxidant activity. In general, extracts with high antioxidant activity show a high phenolic content. Plant extracts with high phenolic contents also show high flavonoid content as reported for other plant species (Makepeace *et al*., 1985). The antioxidant activities of different extracts of pods of *Caesalpinia pulcherrima* are in accordance with their amount of phenolics contents.

CONCLUSION

Currently there has been an increased interest worldwide to identify antioxidant compounds from plant sources which are pharmacologically potent and have small or no side effects for use in protective medicine and the food industry. Increasing acquaintance in antioxidant phytoconstituents and include them in daily uses and diet can give sufficient support to human body to fight those diseases. Brine shrimp lethality assay is very useful and inexpensive way of assessing the bioactivity of plant extracts. The promising result displayed by the plant extract in brine shrimp lethality test justified the efficacy of the plant in traditional medicine. Phytochemical analysis revealed the presence of tannins, flavonoids, steroids and alkaloids. This study affirms the *in vitro* antioxidant potential of crude methanolic, chloroform and petroleum ether extracts of the pods of *Caesalpinia pulcherrima*, with results comparable to those of the standard compounds such as ascorbic acid.

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