RESEARCH ARTICLE

Studies on phytochemical constituents and antioxidant activity of *Alstonia scholaris*

Mistry Dhruti, Parekh Bhavika and Pithawala Meonis*

C G Bhakta Institute of Biotechnology, UKA Tarsadia University, Gopal Vidyanagar, Bardoli Mahua Road, Tarsadi-394350, Gujarat, India
*Address for correspondence: meonis_pithawala@yahoo.com | 09825660930

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ABSTRACT

The study aimed to evaluate and compare qualitatively and quantitatively phytochemical constituents present in bark, stem and leaves of the medicinal plant *Alstonia scholaris*. Qualitative phytochemical screening revealed the presence of alkaloids, saponins, terpenoids, flavonoids, phenolic compounds, tannins, steroids, and glycosides in bark, stem and leaf extracts. The glycosides, alkaloids, gums and mucilage were found in higher quantity in bark of *A. scholaris* as compared to that in stem and leaf. Further, *in vitro* antioxidant potential of extracts from bark of *Alstonia scholaris* was also analyzed. Both aqueous and/or methanolic extracts from bark of *A. scholaris* showed potent total antioxidant activity. At every concentration studied, percentage of superoxide radicals scavenged by aqueous extracts from bark of *A. scholaris* was higher even than those of standard gallic acid at respective concentrations. Similarly the results of DPPH free radical scavenging assay showed that aqueous extracts from bark of *A. scholaris* better scavenged free radicals than the methanolic extract from bark of *A. scholaris* as well as standard Ascorbic acid tested at respective concentrations.

Keywords: *Alstonia scholaris*, phytochemicals, antioxidant activity

INTRODUCTION

Plant-derived substances are of great interest owing to their versatile applications. Plants serve to human the valuable components of medicines, seasonings, beverages, cosmetics and dyes (Bhanu et al., 2013). Most of the drugs for folk medicines, traditional systems of medicine, as well as modern medicines, are derived from plants. From more than 2, 50,000 known species of higher plants, only 5-10% has been chemically investigated (Anushia et al., 2009). Hence it is demanding need of the hour to study the various pharmacologically valuable aspects of many medicinal plants.

An antioxidant can be broadly defined as any substance that delays or inhibits oxidative damage to a target molecule (Yamagishi and Matsui, 2011). Antioxidants regulate various oxidative reactions naturally occurring in tissues and are evaluated as a potential anti-aging agent. There has been increased interest in natural antioxidants, especially those of plant origin. In the past decade there have been many reports of plant extracts and different types of phytochemicals particularly polyphenols, the secondary metabolites from plants, which were shown to have antioxidant activity (Bergman et al., 2001; Sahreen et al., 2010).

Alstonia scholaris (Family: Apocynaceae) is popularly known as "Saptaparni" or "Devil's tree". It is widely distributed in dried forests of India, Western Himalayas, and Western Ghats as well as in the southern India (Nadkarni, 1976). It is a medium to large tree, about 40 m high with a corky grey to grey-white bark. The outer blaze is cream to yellow with abundant, milky latex that flows rapidly when cut. Leaves are in whorls of 4-8 in the upper exiles, upper surface is dark green, the lower green-white. The tip of the leaf is rounded or shortly pointed and tapered towards the base (Meena et al., 2011). Greenish white flowers are umbrellately branched. They are 7-10 mm long.

Alstonia scholaris is a well-known remedy for the treatment of various types of disorders in the folk and Ayurvedic system of Indian medicine. It has been reported to possess antimalarial (Gandhi and Vinayak, 1990; Keawpradub et al., 1999), antimicrobial (Misra et al., 2011; Singh and Sangwan, 2011), free radical scavenging and antioxidant (Arulmozhi et al., 2007; Ravi Shankar et al., 2008; Kumar et al., 2010; Ramachandra et al., 2012), anti-diabetic (Anurakkun et al., 2007; Arulmozhi et al., 2010; Bandawane et al., 2011), analgesic and anti-inflammatory (Arulmozhi et al, 2012; Karawya et al., 2010), anticancer and cytotoxicity (Kamarajan et al., 1991; Keawpradub et al., 1997; Saraswathi et al., 1998; Jagetia and Baliga, 2006; Sharma et al., 2010; Surya Surendren et al., 2012), radioprotective (Gupta et al., 2008; Gupta et al., 2010; Chakraborty et al., 2011), CNS activity (Arulmozhi et al., 2008; Kulkarni and Juvekar, 2009), immunostimulating (Lin et al., 1996; Iwo et al., 2000), antifertility (Gupta et al., 2002), antidiarrheal (Patil et al., 1999; Shah et al., 2010), bronchodilatory (Channa et al., 2005), anti-tussive and anti-asthmatic (Singh et al., 2010) activities.

In one of the studies carried out in our laboratory (Mistry and Pithawala awaiting publication) where *in vitro* cultured human lymphocytes when treated either alone with *Alstonia scholaris* bark, stem or leaf extracts as well as in combination with radiomimetic drug Bleomycin, positive implications about protective effects by *Alstonia scholaris* bark (but not leaf and stem) extract on Bleomycin induced cytogenetic alteration in the form of chromosomal aberrations were recorded. The evidence collected here gave reason to believe that the extracts from bark (but not stem and leaf) of plant *Alstonia scholaris* possess a radio-protective potential. In order to know probable compounds within bark extracts responsible for radioprotection the primary aim of present study was to assess and compare qualitatively and quantitatively phytochemical constituents present in bark, stem and leaves of this medicinal plant.

The superoxide (O_2^-) and hydroxyl (OH.) radicals produced from oxygen and the radio-hydrolysis of water are responsible for most of the DNA and lipid membrane injury caused by radiation. Superoxide dismutase (SOD) and catalase (CAT) are intracellular enzymes that scavenge the superoxide and hydroxyl radicals respectively. Radioprotectors are extracellular, compounds that are designed to reduce the damage in normal tissues caused by radiation. These compounds are often antioxidants and must be present before or at the time of radiation for their effectiveness (Maurya and Devasagayam, 2011). Further many compounds with free radical scavenging activities can be effective radioprotectors. Anti-oxidants are known to scavenge free radicals, thereby decreasing harmful effects of radiation. As bark of the plant and not stem or leaf showed potent radio-protective property (Mistry and Pithawala awaiting publication), an additional study was initiated to analyze *in vitro* antioxidant and free radical scavenging potential of extracts from bark of *Alstonia scholaris*.

MATERIAL AND METHODS

Plant material:

The bark, stem and leaves of *Alstonia scholaris* Linn. R.Br. (Apocynaceae) were collected after proper

identification and authentication. The average age of the plant samples collected was more than 50 years.

Extraction methods:

Alstonia scholaris bark, stem and leaves were collected separately and dried at room temperature. After drying, they were ground crumbly and the extracts were prepared by refluxing with double distilled water (DDW) and methanol as solvents in Soxhlet extractor. The extracts were allowed to cool and concentrated by evaporating their liquid contents and freeze dried. Extracts were re-dissolved in sterile pyrogen free DDW prior to use.

Phytochemical Screening:

Qualitative screening of bioactive compounds present within bark, stem and leaf of *Alstonia scholaris* was carried out following standard protocols.

Tests	Method
Alkaloids	**Wagner's test** The plant extract was added in test tube. Few drops of Wagner's reagent were added along sides of test tube. A reddish brown precipitate was formed which confirmed the presence of alkaloids. **Dragendorff's test** The plant extract was mixed with few drops of acetic acid followed by Dragendorff's reagent. Orange red precipitates were formed indicating the presence of alkaloids.
Flavonoids	5 ml of diluted ammonia solution was added to aqueous filtrate of the plant extract followed by the addition of concentrated sulphuric acid. A yellow coloration was observed in the extract which indicated the presence of flavonoids.
Amino acid (Ninhydrin test)	Drops of Ninhydrin reagent were added to the plant extract, purple colour appeared which confirmed the presence of amino acids.
Carbohydrates (Fehling's test)	The plant extract was boiled in water bath at 60^0C in a test tube. 5 ml of Fehling's solution was added in the test tube. A red precipitate formation indicated the presence of carbohydrate.
Phenolic compound (Ferric chloride's test)	The plant extract was mixed with 2 ml of distilled water. Few drops of 5 % ferric chloride were added along the walls of the test tube. A dark green colour showed the presence of phenolic compound.
Terpenoids (Salkowaski test)	1 ml of chloroform was added to plant extract followed by few drops of concentrated sulphuric acid. A reddish brown precipitate indicated the presence of terpenoids.
Cardiac glycosides (Keller-Kiliani test)	The plant extract was dissolved in 5 ml of glacial acetic acid. Few drops of 5% ferric chloride were added followed by few drops of concentrated sulphuric acid. A greenish blue colour indicated the presence of glycosides.
Oils and Fats (Spot's test)	A drop of extract was pressed between two filter papers. Oil stain on the paper indicates the presence of fixed oil and fats.
Steroids and Sterols (Lieberman's test)	1 ml plant extract was mixed with 10 ml of chloroform and conc. sulphuric acid was added along the sides of the test tubes. The lower sulphuric acid fraction turned yellow with green fluorescence and the upper layer turned red that indicated the presence of steroids and sterols.
Saponins (Foam's test)	The plant extract was mixed with 5 ml of distilled water and shaken vigorously for 10 minutes. Foam formation confirmed the presence of saponins.
Tannins (Ferric chloride test)	The plant extract was mixed with 5 % ferric chloride solution. The formation of blue, blue-black or brownish green coloration indicated the presence of tannin.
Gums and Mucilage	The plant methanolic extract (200 µl) was mixed with 5ml distilled water and 2-3 drops of ruthenium red solution. A pink color formation indicated the presence of gums and mucilage.
Vitamins	The plant extract was treated with dinitrophenyl hydrazine and concentrated sulphuric acid. The formation of yellow precipitate suggested the presence of vitamins

The quantitative analysis of phytochemicals were carried out using standard procedures for determination of total alkaloids (Harborne, 1998), flavonoids (Atanassova et al., 2011), phenolic compounds (Ranalli et al., 2006), steroids (Mann et al., 2010), tannins (AOAC, 2016), saponins (Poornima and Ravishankar, 2008), Terpenoids (Ferguson, 1956) and glycosides (Harborne, 1998; Mosa, Practical course).

Antioxidant Potential:

a) *Total antioxidant activity* (Prieto et al., 1999):
The total antioxidant capacity of the extracts was evaluated by the Phosphomolybdenum method. *Alstonia scholaris* bark extract (0.3 ml) was combined with 3 ml of reagent solution (0.6 M Sulfuric acid, 28 mM Sodium Phosphate and 4 mM Ammonium Molybdate). The tubes containing the reaction solution were incubated at 95oC for 90 min. Then the absorbance of the solution was measured at 695 nm using a UV-VIS spectrophotometer against blank after cooling to room temperature. Methanol (0.3 ml) in the place of extract was used as the blank. The total antioxidant activity was expressed as the number of grams equivalent of ascorbic acid. The calibration curve was prepared by mixing ascorbic (50, 100, 150, 200, 250 and 300 µg/ml) with methanol.

b) *Superoxide anion scavenging activity* (Nishikimi et al., 1972):
The activity was tested using Nitroblue Tetrazolium (NBT) method. The reaction mixture consisted of 1 ml of NBT solution (156 µM) and sample solution at different concentrations (50, 75, 100, 125, 150, 175, 200 and 225 µg/ml). The reaction was started by adding 100 µl of Phenazine Methosulfate solution (60 µM PMS) in phosphate buffer (pH 7.4) to the reaction mixture followed by incubation at 250oC for 5 min and the absorbance was measured at 560 nm against blank. Gallic acid was used as the standard. The percentage of super oxide anion scavenging was calculated as:

% of superoxide anion scavenged = [(Ablank−ASample) /Ablank] × 100

Where:
Ablank = Absorbance of the blank in absence of sample,
ASample = Absorbance in the presence of sample.

a) *Free radical scavenging activity* (Shimada et al., 1992):
Free radical scavenging activity was evaluated using Ascorbic acid as standard antioxidant. The radical scavenging activity was measured using the stable radical DPPH. Various concentrations (2, 4, 8, 16, 32, 64 and 128 µg/ml) of the extracts were added to 4 ml of a 0.004 % methanol solution of DPPH. The mixture was shaken and left for 30 min at room temperature in the dark, and the absorbance was then measured with a spectrophotometer at 517 nm. All determinations were performed in triplicate. The antioxidant activity was calculated as the percent inhibition caused by the hydrogen donor activity of each sample according to the following formula:
Inhibition (%) = (1 − Absorbance of the sample/Absorbance of the Blank) × 100

RESULTS AND DISCUSSION

Qualitative phytochemical screening (Table 1) revealed the presence of alkaloids, glycosides, flavonoids, saponins, terpenoids, gums and mucilage as well as oils and fats in bark, stem and leaf extracts. Quantitative tests revealed that glycosides, alkaloids, gums and mucilage were present in higher quantity (Table 2) in bark of *A. scholaris* but not in leaf and stem.

Both aqueous as well as methanol extracts from bark of *A. scholaris* showed potent total antioxidant activity (Figure 1). Particularly at higher concentrations (200, 250 and 300 µg/ml), the antioxidant activity of aqueous extract was greater than that of standard Ascorbic acid.

From the results of NBT reduction method (Figure 2) it is clear that there is linear increase in percentage of superoxide radicals scavenged by aqueous as well as methanolic extracts from bark of *A. scholaris.* At all concentrations studied, percentage of superoxide radicals scavenged by aqueous extracts from bark of *A. scholaris* was higher even than those of standard Gallic acid at respective concentrations. Similarly the results of DPPH free radical scavenging assay (Figure 3) showed that aqueous extracts from bark of *A. scholaris* better scavenged free radicals than the methanolic extract from bark of *A. scholaris* as well as standard ascorbic acid tested at respective concentrations.

Table 1: Phytochemical studies of *Alstonia scholaris*

Phytochemicals	Bark		Stem		Leaves	
	Aqueous	**Methanolic**	**Aqueous**	**Methanolic**	**Aqueous**	**Methanolic**
Alkaloids	**Present**	**Present**	**Present**	**Present**	**Present**	**Present**
Glycosides	**Present**	**Present**	Absent	Absent	Absent	Absent
Flavonoids	**Present**	**Present**	**Present**	**Present**	**Present**	**Present**
Phenolic compounds	Absent	Absent	Absent	Absent	Absent	**Present**
Carbohydrates	Absent	**Present**	Absent	**Present**	**Present**	**Present**
Tannins	Absent	Absent	Absent	Absent	**Present**	**Present**
Proteins and Amino acids	Absent	Absent	**Present**	Absent	Absent	Absent
Steroids and Sterols	Absent	Absent	**Present**	**Present**	**Present**	**Present**
Saponins	**Present**	**Present**	**Present**	**Present**	**Present**	**Present**
Oils and Fats	**Present**	**Present**	**Present**	**Present**	Absent	Absent
Gums and Mucilage	**Present**	**Present**	Absent	Absent	Absent	Absent
Terpenoids	**Present**	**Present**	**Present**	**Present**	**Present**	Absent
Vitamin	Absent	**Present**	Absent	**Present**	Absent	**Present**

Table 2: Quantitative comparison of phytochemical constituents present in bark, stem and leaf of *Alstonia scholaris*

Phytochemicals	Bark		Stem		Leaf		Statistic F value*
	Aqueous Extract	Methanolic Extract	Aqueous Extract	Methanolic Extract	Aqueous Extract	Methanolic Extract	
Total Alkaloid (% w/w)	7.26 ± 0.06	9.07 ± 0.08	5.08 ± 0.19	4.91 ± 0.06	3.94 ± 0.12	2.82 ± 0.03	128.37
Total Saponin (% w/w)	0.87 ± 0.16	0.24 ± 0.02	0.66 ± 0.11	0.69 ± 0.01	0.91 ± 0.06	0.49 ± 0.01	14.74
Total Terpenoids (% w/w)	14.38 ± 0.57	19.46 ± 0.08	15.30 ± 0.70	17.00 ± 0.05	10.56 ± 0.37	16.23 ± 0.21	7.35
Total Flavonoids (mg/gm dry wt)	37.51 ± 0.04	26.38 ± 0.10	38.56 ± 0.01	40.77 ± 0.06	68.73 ± 0.003	76.23 ± 0.02	111.93
Total Phenolic compounds (mg/gm dry wt)	46.87 ± 0.10	49 ± 0.08	31.25 ± 0.09	35.63 ± 0.02	32.45 ± 0.11	35.10 ± 0.07	6.83
Total Tannin (mg/gm dry wt)	18.70 ± 0.01	14.40 ± 0.003	13.60 ± 0.004	18.64 ± 0.02	11.48 ± 0.01	11.22 ± 0.007	6.15
Total Steroids (mg/gm dry wt)	122.29 ± 0.01	120.89 ± 0.01	115.81 ± 0.01	109.28 ± 0.008	125.13 ± 0.02	122.71 ± 0.01	1.65
Total Glycosides (mg/gm dry wt)	6.29 ± 0.001	3.39 ± 0.005	0.26 ± 0.001	0.21 ± 0.002	0.16 ± 0.002	0.29 ± 0.0008	40
Gums and Muscilage (mg/gm dry wt)	4.31 ± 0.03	1.81 ± 0.06	0.37 ± 0.03	0.02 ± 0.03	00	00	121.93

* One way ANOVA analysis

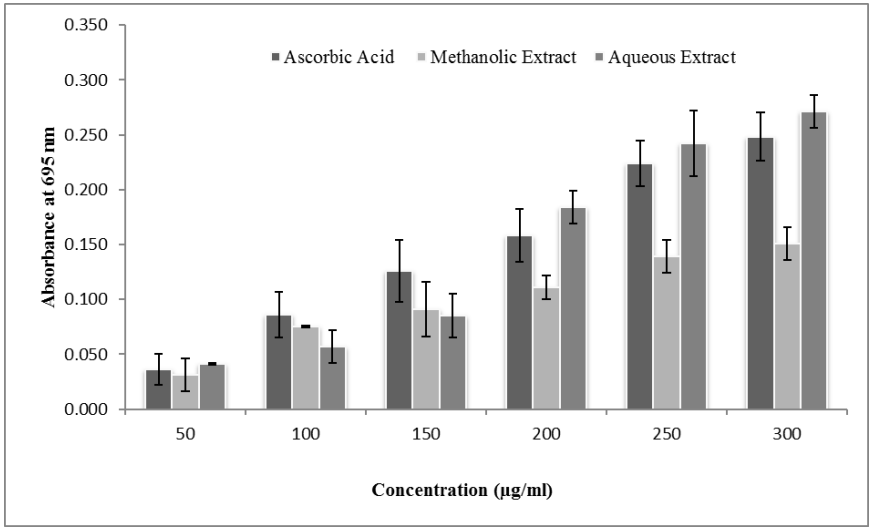

Figure 1

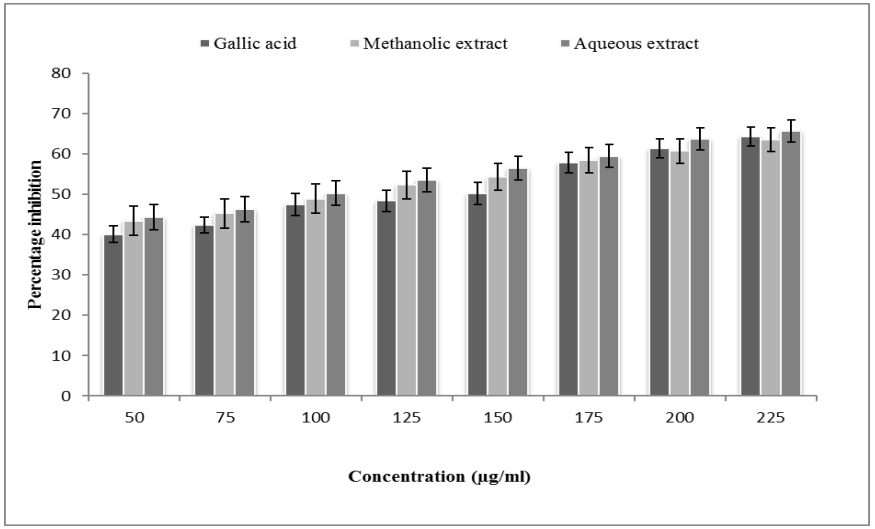

Figure 2

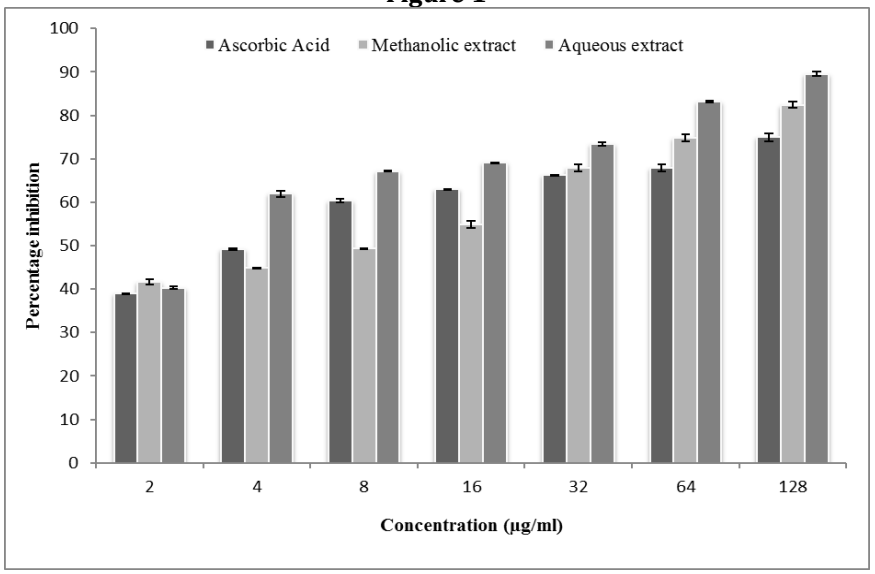

Figure 3

Figure 1: Total antioxidant activity of aqueous and methanolic extracts from bark of *A. scholaris* measured by Phosphomolybdenum method (Ascorbic acid used as standard)

Figure 2: Percentage of superoxide radicals scavenged by aqueous and methanolic extracts from bark of *A. scholaris* using Gallic acid as standard (NBT Reduction Method)

Figure 3: Percentage of free radicals scavenged by aqueous and methanolic extracts from bark of *A. scholaris* using Ascorbic acid as standard (DPPH scavenging method)

The primary aim of present study was to assess and compare qualitative and quantitative phytochemical constituents present in bark, stem and leaves of *A. scholaris*. Glycosides, alkaloids, gums and mucilage were found in higher quantity in bark but not in leaf and stem of *A. scholaris*. In 2011, the phytochemical constitution and antioxidant activity of various solvent (aqueous, butanol and ethyl acetate) extracts of the leaf and bark of *Alstonia scholaris* were investigated (Antony et al., 2011). The authors reported the presence of tannins, proteins, phenols and steroids in aqueous extracts of both leaf and bark. Our findings are consistent with their results.

Earlier *in vitro* antioxidant and free radical scavenging activities of *A. scholaris* have been reported (Arulmozhi et al., 2007). However, the authors have used leaves of the plant and not bark. In their study ethanolic extracts from leaves of *Alstonia scholaris* had significant DPPH free radical scavenging, metal ion chelating, hydrogen peroxide scavenging, superoxide anion radical scavenging and ferric thiocyanate reducing activities. Further, the ethanolic extract of leaves of *Alstonia scholaris* had significant *in vitro* antioxidant activity as well.

In our study total antioxidant activity, superoxide radicals scavenging and DPPH free radical scavenging assay were performed using aqueous and methanol extracts from bark of *A. scholaris*. Results revealed that aqueous extracts had better potency for antioxidant activity particularly at higher concentrations as compared to methanol extracts. The bark extracts also showed superoxide and DPPH free radical scavenging activities. Similar findings were reported (Antony et al., 2011) earlier, where significant antioxidant activity was observed with DPPH, ABTS and FRAP assays from aqueous and butanol extracts as compared to the ethyl acetate extract.

Plants containing large amounts of antioxidants, such as polyphenols, vitamin C, vitamin E, selenium, β-carotene, lycopene, lutein, and other carotenoids neutralize free radicals, quench singlet and triplet oxygen, or decompose peroxides (Djeridane et al., 2006). Flavonoids have been attracted a great deal of attention due to their potential health benefits. Several experimental studies (Robak and Marcinkiewiez, 1995; Parker et al., 1999) have demonstrated antioxidant effects, which are associated with free radical-scavenging action of flavonoids. Further it has been demonstrated (Willis et al., 1996) that flavonoids protect cells against oxidative damage and reduce the risk of developing certain types of cancers.

The bark of *A. scholaris* is the most intensively used part of the plant and is used in many compound herbal formulas (Nadkarni, 1976). In the present study, the qualitative and quantitative analysis of extracts from bark of *Alstonia scholaris* revealed presence of flavonoids. Additionally, the quantity of terpenoids was highest in aqueous extracts as compared to other compounds followed by steroids, alkaloids and phenolic compounds. It has been reported that plant secondary metabolites such as flavonoids and terpenoids play an important role in defence against free radicals (Govindarajan et al., 2005). The free radical scavenging and antioxidant potential of *A. scholaris* observed in the present finding could be attributed to terpenoids, steroids, alkaloids, flavonoids and phenolic compounds. Thus antioxidant and free radical scavenging potential of the plant so exhibited could be responsible for radioprotective potentials.

Plants that produce antioxidants which scavenge free radicals caused by radiation, exhibit radio protective action. The results of our present study add to this body of knowledge one more plant (*Alstonia scholaris*) with such potentials.

Conflicts of interest: The authors declare no conflicts of interest

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