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Phytochemical and antioxidant activity of methanolic leaf extract of *Alstonia scholaris* Linn.

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Abstract

Alstonia scholaris is a plant of the Apocynaceae family and as great medicinal important. It is widely used by triable people to treat various for the treatment of diseases and ailments such as antimicrobial, anticancer, anti-inflammatory, anti-infertility and wound healing activities. The *Alstonia scholaris* leaves was collected wash with saline water, shade dried room temperature for 20 days. The dried plant materials was pulverized into fine powder using a grinder (mixer). About 9gm leaf powder was extracted in soxhlet apparatus with 150ml methanol. Crude extract obtained is subjected to column chromatography for purification of active compounds. The individual fractions were subjected to qualitative the identification of the active compounds. Methanol extract and toluene : chloroform (80:20) revealed the presence of the presence of alkaloids, flavonoids, carbohydrate, phenolos, steroids, saponins and tannins, amino acids, terphenoids the quantification of total alkaloids, total phenols, total flavanoids, and total tannin has been identified leaves of crude extract and column purified samples by spectrophometer. The present study identify with standardization of the plant leaves of various phytoconstituents and antioxidant potential with column purified compounds were free radical scavenging assay of 2,2 -diphenyl-1-picryl-hydrayl (DPPH),SO & ABTS in *in vitro* assay. The present study revealed methanol leaf extract of *Alstonia scholaris* would exert several beneficial effects by virtue antioxidant activity and need further investigations for it to be use in clinics in the future.

Keywords: *Alstonia scholaris* antioxidant potential, phytochemical constituent

Introduction

Medicinal plants are a sources of great economic value all over the world. (Maobe, A.G *et al.* 2013) ^[13] plants are rich source of novel drugs that forms the ingredients in traditional systems of medicine. ((Ncube, 2008) ^[19] It has been used in treatments for numerous human diseases for therapeutic aid for alleviating the ailments (Momin RK, and Kadam VB; 2011) ^[17] Phytochemicals are bioactive chemicals of plant origin. (Pan dith *et al.*, 2012). Secondary metabolites are synthesized by the plants as part of the defence system of the plant (Phan TT *et al.*, 2001) ^[22] Natural antioxidants, which are to block the process of oxidation by neutralizing free radicals (Mojab F 2003) ^[15] The oxidation induced by reactive oxygen species (ROS) in cell membrane disintegration, membrane protein damage and DNA mutation, the development of many disorders, such as cancer, liver injury and cardiovascular diseases (Liaoand Yin, 2000) ^[10] These reactive species can react with biomolecules, causing cellular injury and death (Valko M, *et al* 2007) ^[31]

Alstonia scholaris belongs to family Apocynaceae and is native to India. (Khare, 2007) ^[8] It grows India, in deciduous and ever-green forests plains. (Kirtikar KR, 2002) This plant is used in traditional, (Rahmatullah *et al.*, 2009) ^[23] Ayurvedic, Unani and Homeopathy types of alternative medicinal systems against different ailments such as fever, diarrhoea, asthma, and lung cancer of phytochemistry and pharmacological activities (Wiar C. 2006) ^[34] Juice of leaves acts as a powerful used in snake bite. (Joshi SG, 2000) ^[5] Milky juice of the plant is applied on wounds and ulcers (Nadkarni *et al.* 1976) ^[18] It is useful leprosy, skin diseases, chronic bronchitis. (Uniyal MR, 1991) ^[30] The bark useful in malarial treatment (Sedlack J 1968) ^[26] The ripe fruits are used to treat insanity due to syphilis and epilepsy (Baliga *et al* 2010) ^[2] It has been reported as antimicrobialanti-cancer (Kamarajan *et al.* 1991) ^[6] anti-inflammatory and, antioxidant and anti-fertility (Misra CS *et al.* 2011)) ^[14] tumour, jaundice, hepatitis and, skin diseases (Mollik *et al.*, 2010) ^[16] anti-diarrhoeal (Patil *et al* 1999) ^[21] mental disorders, cardiopathy, helminthiasis, pruritus, agalactia (Singh and Sangwan, 2011) ^[28], anti-mutagenic effect(Lim-Syliano C.Y. *et al.*, 1990) ^[11] The methanolic extract of this plant was found to be antiplasmodial activity (Keawpradup N. *et al.*,1999) ^[7] *Alstonia scholaris* (Linn) is a rich source of alkaloids (about 180 alkaloids) isolated, so far only few have been assessed for biological activities. (Versha P, B. 2003) ^[32] The alkaloid fraction of *A. scholaris* was found to

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have potential anticancer agent (Jagetia GC, Baliga MS: 2006) [4] It contain various types of alkaloids, steroids, triterpenoids, flavonoids and phenolic acids (Arulmozhi S, *et al.* 2007) [1]



Fig 1: *Alstonia scholaris* Linn

Materials and Methods

Collection of plant materials

The fresh leaves of *A. scholaris* were collected in the month of October (2016) from Nasiyanoor (Vill), Erode (Dt).

Extraction by Soxhlet Method

The leaves of *A. Scholaris* was collected, washed with running tap water, shade-dried at room temperature for 20 days. The dried plant material was pulverized into fine powder using a grinder (mixer). About 9 g of powdered material was extracted in soxhlet extraction apparatus with 150 ml of methanol (Vogel, 1988) [33]. The solvents were evaporated (at 40°C) with the help of heating mantle. The greenish substances were obtained and stored room temperature evaporated to dryness and percentage yields were calculated (Beyer and Walter, 1997).

Column chromatography for isolation & purification

Column chromatography is commonly used purification technique isolation of pure, phamocologically active constituents from plants remain a long and tedious process. It is necessary to have methods available for efficient separation from plant extracts, to isolate the purify compound. The column chromatography (length: 450mm; Bore: 30mm) was performed using 60 – 120 mesh silica gel to elute out individual components from the crude plant extract. The column was rinsed with hexane and completely dried before use. The column was filled 3/4th with mixture of solvents ratio (1:2:0.5) (Distilled water: Acetic acid: Chloroform) and 20g of silica gel was packed 2/3rd of the column length with simultaneous draining of the solvent to aid proper packing. The packing was performed after activating the silica gel gently poured on the top of the column with constant tapping to avoid air bubbles and cracks after mixing with solvents. Add 2mm layer of sea sand into the top of the column after

settled the gel. The column was run with solvent of (Toluene: Chloroform 8:2) after loading with the crude plant extract (2-3g) mixed with activated silica gel. The fractions collected were dried for further analysis (fig-2)

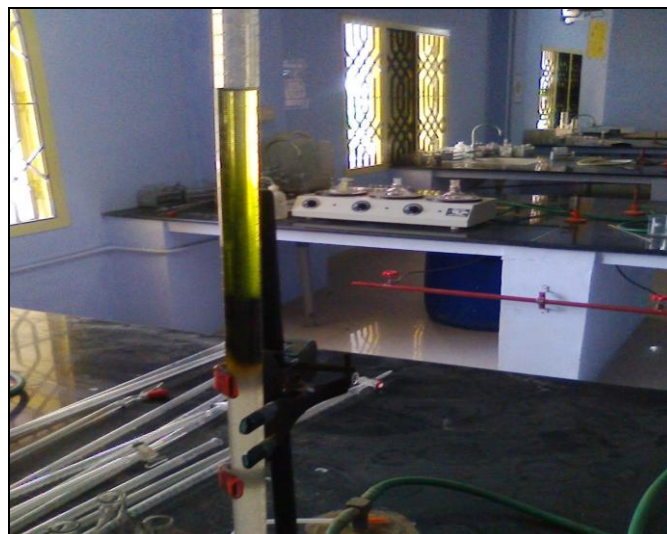


Fig 2: column chromatography

Qualitative Phytochemical Analysis (Edeoga *et al.*, 2005) [3]

Phytochemical screening was performed to identification of phytochemical in the crude extract and column purified sample were carried out by standard procedure in the different solvents are Hexane, Ethanol, methanol, chloroform, Ethyl acetate and water.

Tests for Alkaloids

- a. **Dragendorff's Test:** 0.5 g of the extract was stirred with 5 ml of 1% aqueous hydrochloric acid on a water bath and filtered. 3 ml of the filtrate was divided into 3 parts. To the first 1 ml, few drops of freshly prepared Dragendorff's reagent was added. An orange to brown precipitate indicates the presence of alkaloids.
- b. **Mayer's Test:** To the second part, 1 drop of Mayer reagent was added. White to yellow or cream colour precipitate indicates the presence of alkaloids.
- c. **Wagner's Test:** To the third part, Wagner reagent was added. Formation of brown or red or reddish-brown precipitate indicates the presence of alkaloids.

Tests for Carbohydrates

- a. **Molisch's Test:** The extract was treated with few drops of alcoholic alpha naphthol Solution. Then 0.2 ml of concentrated sulphuric acid was added slowly through the sides of the test tube. A purple to violet colour ring appears at the junction indicates the presence of carbohydrates.
- b. **Benedict's Test:** The extract was treated with few drops of Benedict's reagent (alkaline solution containing cupric citrate complex) and upon boiling on water bath. Presence of reddish brown precipitate indicates the presence of reducing sugars.
- c. **Fehling's Test:** Equal volume of Fehling's A (Copper sulphate in distilled water) and Fehling's B (Potassium

tartarate and Sodium hydroxide in distilled water) reagents are mixed and few drops are added to the extract and boiled. A brick red precipitate of cuprous oxide forms, if reducing sugars are present.

Test for Tannins

- a. Ferric chloride Test:** 0.5 g of extract was stirred with 10 ml of distilled water and then filtered. Few drops of 1% ferric chloride solution were added to 2 ml of the filtrate. Occurrence of a blue-black, green or blue-green precipitate indicates the presence of tannins.

Test for Terpenoids

- a. Salkowski Test:** Five ml of extract was mixed in 2 ml of chloroform. Concentrated sulphuric acid (3ml) was carefully added to form a layer. A reddish brown coloration of the interface indicates presence of terpenoids.

Test for Saponins

- a. Frothing Test:** 0.5 gm of the extract was shaken with distilled water in a test tube. Frothing which persists for 15 minutes indicates the presence of saponins.

Tests for Flavonoids

- a. Shinoda Test:** Three pieces of magnesium chips were added to an alcoholic solution of the extract. Then few drops of concentrated hydrochloric acid was added. Appearance of an orange, pink or red to purple colour indicates the presence of flavonoids.
- b. Ferric Chloride Test:** The extract was boiled with water and filtered. Two drops of freshly prepared 10% ferric chloride solution was added to 2 ml of filtrate. Green, blue or violet colour indicates the presence of phenolic hydroxyl group.
- c. Sodium Hydroxide Test:** 2 ml of alcoholic solution of extract was dissolved in 10% aqueous sodium hydroxide solution and filtered to give yellow colour. Change in colour from yellow to colourless on addition of dilute hydrochloric acid indicates the presence of flavonoids.
- d. Lead Acetate Test:** 10% lead acetate solution was added to the aqueous solution of the extract. Yellow precipitate indicates the presence of flavonoids.

Test for Steroids

Liebermann Burchard Test: 2 ml of diluted sulphuric acid was added to 0.5 g of crude extract dissolved in 2 ml of acetic anhydride. Formation of a blue or green solution indicates the presence of steroids.

Quantitative analysis

Determination of total phenolic content

Total phenolics in extracts were determined by Folin-Ciocalteu method (Ranalli *et al.*, 2006) [24]. Briefly, 0.5 mL (0.1%, w/v) of each sample was mixed with 2.5 ml of a 10 fold diluted Folin- Ciocalteu reagent followed by 2 ml of 7.5% sodium carbonate. The tubes were covered with parafilm (American National Can, Chicago) and allowed to stand for 30 min at room temperature before the absorbance was recorded at 760 nm (U-1800, Spectrophotometer, Hitachi, Japan). Different concentrations of gallic acid (0.1 to 0.60 mg/mL) were prepared in methanol for preparation of

standard curve. All determinations were analyzed in triplicate and results expressed in mg gallic acid equivalents (GAE)/g dried extract. (Ranalli *et al.* (2006) [24])

Determination of total flavonoid content

The total flavonoid content of plant extracts were estimated according to method described by (Zhishen *et al.* (1999) [35]). 1.0 ml (0.1%, w/v) of sample was mixed with 4 mL of distilled water and subsequently with 0.3 ml of NaNO₂ solution (10%, w/v). After allowing the mixture to stand for 5 min, 0.3 ml AlCl₃ solution (10%, w/v) was added followed by 2.0 ml of (1%, w/v) NaOH solution. The mixture was thoroughly mixed immediately and absorbance was determined against blank at 510 nm. Standard curve of quercetin (Sigma Aldrich, USA) was prepared in a concentration ranging from 0 to 12 mg/mL and the results were expressed as quercetin equivalents (mg quercetin equivalents/g dried extract).

Determination of tannin

Tannin content in sample was determined using insoluble polyvinyl- polypyrrolidone (PVPP) which binds tannins (Makkar *et al.*, 1993) [12]. Briefly, 1 ml of extract (0.1%, w/v) dissolved in methanol in which the total phenolics were determined, was mixed with 100 mg PVPP, vortexed, kept for 15 min at 4°C and then centrifuged for 10 min at 3000 rpm. In the clear supernatant tannin phenolics were determined. Tannin content was calculated as a phenolic content. 450.

Antioxidant activity

Methanolic crude extract and column purified sample of were analyzed using assay such as DPPH, ABTS&SO for evaluating antioxidant attributes.

DPPH (1,1-Diphenyl-2-picryl-hydrazil.) Free Radical Scavenging Activity: (Shimada *et al.*, 1992) [27]

Various concentrations of plant extracts (20-100 µg/ml) were mixed with 1.0ml of methanolic solution containing DPPH, resulting in the final concentration of DPPH being 0.2mM. The mixture was shaken vigorously, left for 30min, at room temperature and the absorbance was measured at 517 nm. The reaction was initiated by the addition of 1.0ml of diluted DPPH to 10µl of methanol as control. Ascorbic acid, Trolox, BHA (20-100 µg/ml) was used as positive controls. The DPPH radical scavenging activity was calculated as follows: Scavenging activity = $[(A_0 - A_1) / A_0] \times 100$, where A₀ was the absorbance of the control (blank, without extract) and A₁ was the absorbance in the presence of the extract. EC₅₀ value (µg extract/ml) was the effective concentration at which DPPH radicals were scavenged by 50% and were obtained by interpolation from linear regression analysis

ABTS radical scavenging activity (Re *et al.*, 1990)

The plant extracts (20-100 µg/ml) were mixed with 1.0ml of ABTS solution resulting in the final concentration of ABTS being 7mM. The mixture was shaken vigorously; the reaction was initiated by the addition of 1.0ml of diluted ABTS+ to 10µl of methanol as control. The absorbance was read at 734nm after 6min and the percentage inhibition was calculated. Ascorbic acid, trolox, BHA (20-100 µg/ml) was used as positive controls. The inhibition was calculated according to the equation Scavenging activity = $[(A_0 - A_1) / A_0]$

$\times 100$, where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract. EC₅₀ value (μg extract/ml) was the effective concentration at which ABTS radicals were scavenged by 50% and were obtained by interpolation from linear regression.

SO radical scavenging activity (Ock-Sook *et al.* 1997)

4.5ml of 0.05 M Tris HCL buffer pH 8.2 was added in to test tube, followed by adding 1ml of the extract and 0.5ml of 2.5mM pyrogallol solution. The reaction mixture was incubated at 25°C for 5 min and then the reaction was stopped by to addition of 1ml of 8.0 m HCL and the absorbance was measured at 320nm The inhibition was calculated according to the equation Scavenging activity = $[(A_0 - A_1/A_0)] \times 100$, where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract. EC₅₀ value (μg extract/ml) was the effective concentration at which SO radicals were scavenged by 50% and were obtained by interpolation from linear regression.

Results and Discussion

The results reveals that the *Alstonia scholaris* plant shows the presence of phytochemical constituents are Alkaloids, flavonoids, tannins, carbohydrates, amino acids, phenols, steroid and proteins in methanolic crude extract Was showed in (Table.1) The Maximum Concentrations of alkaloids are Observed in methanol and ethanolic extract of Purified sample when compared with other solvents. Similarly other phytochemical showed to be in various solvents at lowest grade. The quantitative analysis of phytochemical presented in (Table.2) methanolic leaf extract of *Alstonia scholaris* crude and column purified sample were obtained that total alkaloids content was found to be high in crude extract 15.52mg/g compared to column purified extract 13.6mg/g. Similarly total flavonoids contents of crude extract 7.81mg ; column purified samples 2.34mg, total tannins contents of crude extract 3.96mg ; column purified samples 2.01mg & total phenol contents of crude extract 2.31mg; column purified samples 0.82mg and respectively.

Table 1: Phytochemical compounds of different solvent column purified samples

Phytochemical Tests	Inference					
	Hexane	Chloroform	Ethyl acetate	Ethanol	Methanol	water
Alkaloids	++	++	++	+++	+++	++
Flavanoids	+	-	+	++	++	+
Tannins	+	-	+	-	-	-
Phenols	+	+	+	+	+	-
Proteins	-	-	-	+	+	-
Cabohydrates	+	+	++	++	++	++
Steroids	+	+	-	+	+	-

Table 2: Quantity of phytochemical compounds in methanolic leaf extract of *Alstonia scholaris*

Phytochemical	Crude extract in mg	Column purified in mg
Total alkaloids	15.52	13.6
Total flavanoids	7.81	2.34
Total tannins	3.96	2.01
Total phenols	2.31	0.82

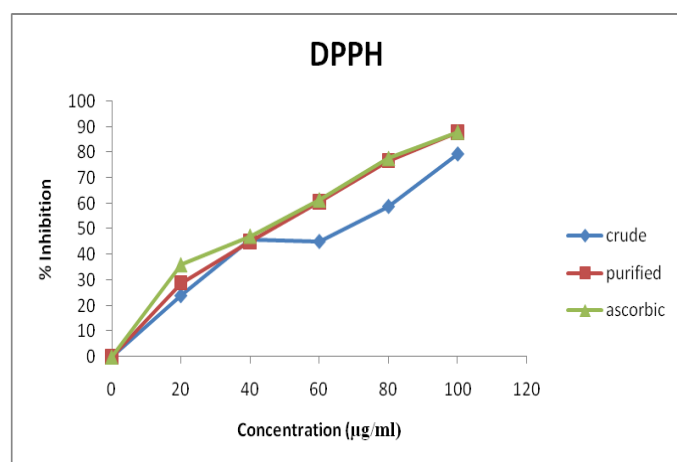


Fig 3: DPPH radical scavenging assay

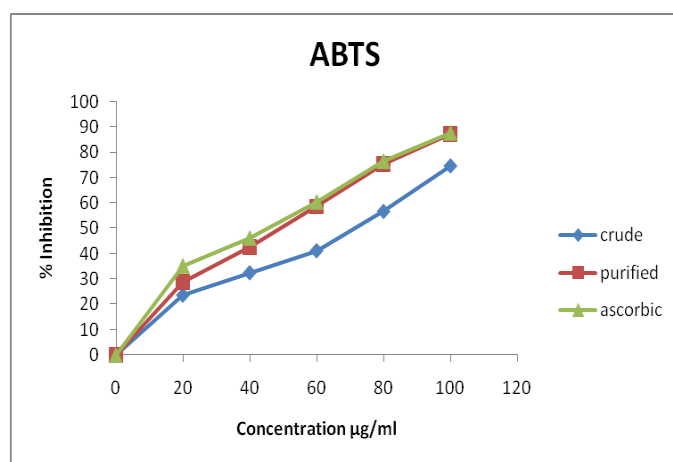


Fig 4: ABTS radical scavenging assay

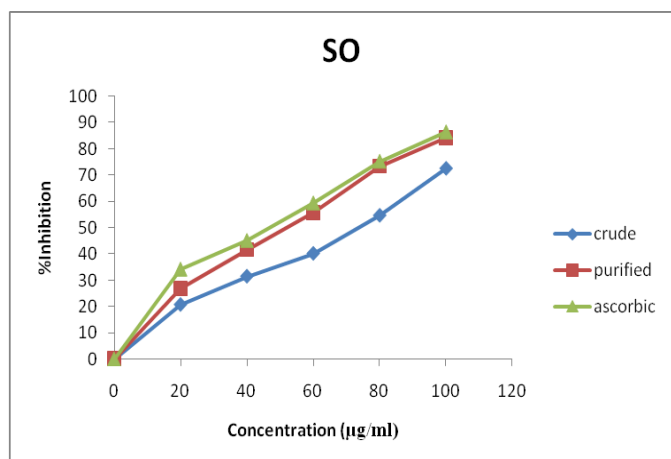


Fig 5: Super oxide radical scavenging assay

The DPPH radical scavenging (%) activity of methanolic leaf extract of *Alstonia scholaris*, and column purified samples were compared to standard ascorbic acid is shown (Fig.1) The IC_{50} value of crude extract was 60.9 µg/ml and column purified samples was 41.64 µg/ml and for ascorbic acid was found to be 37.41 µg/ml The ABTS radical scavenging (%) activity of methanol leaf extract of *Alstonia scholaris*, IC_{50} value of crude extract was found to be 44.48 µg/ml and column purified samples was found to be 49.86 µg/ml and for ascorbic acid was found to be 49.91 µg/ml (Fig.2) The SO radical scavenging (%) activity of methanol leaf extract of *Alstonia scholaris*, IC_{50} value of crude extract was 45.88 µg/ml and column purified samples was 42.87 µg/ml and for ascorbic acid was found to be 38.5 µg/ml (Fig.2) The results indicate that the DPPH leaf extract increased with the increased concentrations. Natural products play vital role in chemotherapy a great percentage of in cancer chemotherapeutic pharmaceutical. (Suzuki I *et al.* 2002)^[29]

Conclusion

The results obtained From the leaves of *Alstonia scholaris* Linn extract revealed that has highest phytochemical constituents and potential antioxidants activity. The present study suggested that the purified methanolic extract of *Alstonia scholaris* Could be a potential source of antioxidants and phytochemical and thus useful as therapeutic agent against oxidative stress related degenerative disease and disorders in future this study extended to isolation, characterization of bioactive compound.

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