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Immuno modulatory and free radical scavenging effects of *Saraca asoca* (Roxb.) W. J. de Wilde stem bark

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Abstract

Saraca asoca (Roxb.) W. J. de Wilde is commonly known as 'Ashoka' tree is one of the most legendary and a sacred tree of India and it is distributed throughout India. The plant has been regarded as a universal panacea in Indian traditional system of medicine, Ayurveda and it has been reported to be used since ancient times. The dried bark and flowers of *S. asoca* is given as tonic to ladies for uterine disorders such as menorrhagia, leucorrhoea, bleeding hemorrhoids, dysfunctional uterine bleeding etc. The present study is aimed to scientifically evaluate the immunomodulatory and free radical scavenging effects of the ethanolic extract of *S. asoca* stem bark. The results showed that the extract exhibited significant immunomodulatory effect in delayed type hypersensitivity reaction (DTH) as evidenced by the increase in footpad thickness of 200 mg/kg treated dose compared to the control. There was a significant increase in the relative organ weights of spleen and thymus were observed in the extract administered group (200 mg/kg). Total WBC count was also increased compared to the control group. The ethanolic extract of *S. asoca* stem bark also stabilized human red blood cell (HRBC) membrane *in vitro* and exhibited significant free radical scavenging effect *in vitro* against DPPH (1, 1-diphenyl-2-picryl hydrazyl) and hydroxyl radicals.

Keywords: Immunomodulation, Delayed type hypersensitivity, HRBC membrane stabilization, Antioxidant, Free radicals

Introduction

Immune functions are crucial for defending against pathogens or malignancies, and thus immune system play an important role in the maintenance of human health. The immune functions are disturbed by factors such as malnutrition, physical and mental stress, ageing and undesirable life- style (Yehia et al., 2011). Immunomodulators are natural or synthetic substances that help to regulate the immune function. They are biological response modifiers which exert their effects by improving host defence mechanisms and maintain a balance between regulatory and effector cells (Agrawal et al., 2010). Medicinal plants are invaluable sources for drug development and there is an ever-growing interest in the field of scientific evaluation of plants to identify their therapeutic efficacy and isolation and characterization of phytocompounds. The increasing interest is due to the tremendous historical legacy of plants using as medicines in traditional/tribal/folk medical practices. Herbal medicines become popular because of their easy availability, cost effectiveness and presumed safety compared to modern synthetic medicine (Guerra et al., 2003).

'Ashoka', is the Sanskrit name of *Saraca asoca* (Roxb.) W. J. de Wilde that means "without sorrow", is one of the ancient medicinal plants grown in India. It is a small evergreen tree growing upto7-10 m height, belonging to the family Caesalpiniaceae. It is distributed mainly in Asia

and some parts of North America and is found throughout India especially in Himalaya, Western Ghats and central areas of Deccan plateau (Murthy et al., 2008). *S. asoca* is a vulnerable plant species and becoming rarer in its natural habitat (Pradhan et al., 2009). Ayurveda texts from the medieval period and till recent times clearly highlight the various therapeutic uses of *S. asoca*. The plant is ethno-botanically used against conditions such as gynaecological problems, mental tranquillity, diabetes, uterine pain etc. (Singh et al., 2015). Ashoka bark is used by women folk for the treatment of menorrhagia, menstrual and uterine disorders in India, Sri Lanka, Pakistan and Bangladesh (Begum et al., 2014). The biological and pharmacological properties reported in *S. asoca* includes antibacterial, anticancer, antimenorrhagic, antioxytocic, antiarthritic, antiulcer, analgesic and cardio-protective effects (Singh et al., 2015). In the present study, the immunomodulatory and *in vitro* free radical scavenging effects of *S. asoca* stem bark ethanolic extract was evaluated.

Materials and Methods

Collection of plant material

The stem bark of *Saraca asoca* was collected from JNTBGRI campus and they were authenticated by the plant taxonomist of the Institute and a voucher specimen (TBGT 57035 dated 16/12/2008) was deposited at the Institute's Herbarium. The stem bark was washed thoroughly, shade-dried and powdered. The powdered plant material was stored at room temperature in airtight containers under dark conditions.

Preparation of plant extracts

50 g of the bark powder was extracted with 500 ml of 95% distilled ethanol for 24 h at room temperature with constant stirring. The extract was filtered and the filtrate concentrated under reduced pressure using rotary evaporator (Buchi, Switzerland) to yield 7.35 g of the crude extract and it was referred to as SA. For *in vivo* studies, SA was reconstituted in 1% Tween-80 (vehicle) to required concentrations.

Chemicals

Levamisole, Dextrose, Sodium citrate, Citric acid, Ascorbic acid, Thiobarbituric acid (TBA), Sodium dodecyl sulphate (SDS), DPPH, 2-deoxy-2-ribose and Ethylene diamine tetra acetic acid (EDTA) were purchased from Sigma Aldrich, USA. All the other chemicals and reagents were of analytical reagent grade.

Animals

Swiss albino mice, males weighing 25-30 g were obtained from the Institute's Animal House. They were housed in polyacrylic cages under standard conditions (temperature 24^0-28^0C, relative humidity 60-70% and 12 h dark-light cycles), fed commercial mice feed (Lipton India Ltd., Mumbai, India) and boiled water *ad libitum*. Animals were acclimatized for one week before starting the experiments. All experiments involving animals were carried out according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India, after getting the approval of the Institute's Animal Ethics Committee (Approval No. B-04/12/2009/EM & EP-06).

Effect of on body weight, lymphoid organ weight and haematological parameters

Swiss albino mice, males weighing 25-30 g were divided into five groups and each group comprised of six animals. Group 1 was control and received normal saline; groups 2-5 received SA at 25, 50, 100 and 200 mg/kg doses respectively for 5 days. The animals were sacrificed by carbon dioxide inhalation, 24 h after the last dose. Body weight gain (%) and relative organ weight of kidney, liver, spleen and thymus were determined for each animal. The effect of the extract on blood parameters (RBC, WBC and Hb) were also estimated (Sharififar et al., 2009).

Delayed type hypersensitivity (DTH) test

Swiss albino mice, males weighing 25-30 g were immunized by injecting 20 µl of 5×10^9 sheep red blood cells (SRBC)/ml subcutaneously into the right hind footpad on day 0 and challenged 7 days later by injecting intradermally the same amount of SRBC into the left hind footpad. Thickness of the left hind footpad was measured with digital Vernier calipers at 24 h and 48 h after challenge. SA in doses of 25, 50, 100 and 200 mg/kg was administered orally on each of the 2 days prior to immunization, on the day of immunization and on each of the days after immunization (days -2, -1, 0, 1 and 2). Mice were immunized on day 0 and challenged on day 7 by sub-plantar injection of 0.1 ml of 5×10^9 SRBC/ml. The volume of the challenged foot was measured at 24 h and 48 h (Doherty, 1981).

Human red blood cell (HRBC) membrane stabilization study in vitro

Blood was collected from healthy volunteer who had not taken any non-steroidal anti-

inflammatory drugs (NSAIDS) for two weeks prior to the experiment. It was mixed with an equal volume of sterilized Alsever's solution consisting of 2% dextrose, 0.8% sodium citrate, 0.05% citric acid and 0.42% sodium chloride in distilled water and it was centrifuged at 3000 rpm for 15 min. The supernatant was discarded and the packed cells were washed with isosaline (0.85%, pH 7.2). The assay mixture contained varying concentrations of SA, 1 ml phosphate buffer (0.15 M, pH 7.4), 2 ml of hyposaline (0.36%) 0.5 ml of HRBC suspension. 2 ml distilled water was used as control. The assay mixture was incubated at 37^0C for 30 min and centrifuged for 10 min at 3000rpm. The absorbance was measured at 560 nm. The percentage hemolysis was calculated by assuming the hemolysis produced in the presence of distilled water as 100%. The percentage of HRBC membrane stabilization or protection was calculated (Gandhidasan et al., 1991) using the formula:

$$\text{Protection (\%)} = 100 - \frac{\text{OD of drug treated sample}}{\text{OD of control}} \times 100$$

DPPH radical scavenging activity

DPPH (1,1-diphenyl-2-picryl hydrazyl) assay has been extensively used for the screening of different plant extracts for free radical scavenging effect, because it can accommodate many samples in a short period and it is very sensitive to detect active ingredients at low concentrations. DPPH radical scavenging activity was measured by the spectrophotometric method. To the methanolic solution of DPPH (200 µM) 0.05 ml of the test compound dissolved in ethanol were added at different concentrations. An equal amount of ethanol was added to the control. After 20 min, the decrease in the absorbance of test mixture due to quenching of DPPH free radicals was read at 517 nm and the percentage inhibition calculated (Sreejayan and Rao, 1996).

Hydroxyl radical scavenging assay

Scavenging of the hydroxyl radical was measured by preparing 200 µl of 2.8 mM 2-deoxy-2-ribose, the test extracts at different concentrations, 400 µl of 200 µM $FeCl_3$, 1.04 mM EDTA (1:1 v/v), 200 µl of 1.0 mM H_2O_2 and 200 µl of 1 mM ascorbic acid. All solutions were freshly prepared. After an incubation period of 1 h at 37°C, the extent of deoxyribose degradation was measured by the thiobarbituric acid (TBA) reaction. The optical density was measured at 530 nm and percentage inhibition was determined (Halliwell and Gutteridge, 1987).

Statistical analysis

All the analyses were carried out in triplicate. The statistical significance between the samples were determined by the analysis of variance (ANOVA) and the data were recorded as mean ± standard deviation (SD) , $P \leq 0.05$ was considered to be statistically significant. Significant differences between means were determined by Dunnett's multiple comparison test (Raghava, 1987). The computer software employed for the statistical analysis was IBM SPSS Statistics, version 20 (USA).

Results

None of the studied doses of *S. asoca* stem bark extract showed toxicity or mortality in the treated groups. No significant difference in the body weight gain were recorded in SA treated groups. The extract did not alter the relative weights of kidney and liver in tested doses, however a significant ($P \leq 0.05$) increase was observed in the relative organ weights of spleen and thymus of 200 mg/kg treated group (Table 1).

There was a significant ($P \leq 0.05$) increase in the total leucocyte count (WBC) was observed in SA 200 mg/kg treated dose ($6.25 \pm 2.8 \times 10^3/mm^3$) compared to the control group. No significant differences in other blood parameters such as RBC and haemoglobin were recorded bewteen extract administered groups.

The cell mediated immune response of SA was evaluated by SRBC induced delayed type hypersensitivity (DTH) reaction in mice and it is reflected in the increased paw edema (Bafna and Mishra, 2004). The footpad thickness reached a peak at 24 h, after which it subsided. SA produced a significant ($P \leq 0.05$), dose dependent increase in DTH reaction in mice compared to the control group. SA (200 mg/kg) exhibited maximum DTH response at 24 h being 0.82 ± 0.010 and 0.66 ± 0.020 respectively (Table 3). The increase in DTH reaction in mice in response to cell dependent antigen (SRBC) indicated the stimulatory effect of the extract on cell mediated immune response.

SA showed dose dependent stabilizing effect *in vitro* towards HRBC compared to the control. All the treated doses (75 µg/ml – 200 µg/ml) exhibited significant ($P \leq 0.05$) protection (above 75%) compared to the control. SA at 200 µg/ ml dose showed 90% membrane stability and 175 µg/ml exhibited 86.43% membrane stability (Table 4).

In DPPH radical scavenging assay, SA exhibited significant ($P \leq 0.05$) radical scavenging potential in a dose dependent manner compared to ascorbic

acid standard (Figure 1). The extract at 500 µg/ ml showed maximum protection against DPPH radicals (79.43%) and IC_{50} value was determined as 36.93 µg/ml. The IC_{50} values are calculated from the standard graph.

The crude ethanolic extract of *S. asoca* stem bark (SA) showed potent hydroxyl radical scavenging activity compared to standard ascorbic acid, whereas 75.88% inhibition was noted with 500 µg/ml of SA. The IC_{50} value of SA was determined as 57.34 µg/ml for hydroxyl radical scavenging assay. Thus it is indicated that SA possesses the ability to scavenge hydroxyl radicals effectively in a dose dependent manner (Figure 2).

Discussion

The immunity has been shown to be suppressed in conditions such as immuno-deficiency diseases, AIDS and cancer. The chemotherapy and radiation therapy in cancer treatment suppress the immune system and any imbalance occuring between regulatory and effector cells of immune system can also cause immunological breakdown and pathogenesis. The immunomodulators can solve these complications to a greater extent (Ramnath et al., 2002).

Synthetic immunomodulators such as levamisole, IL-2, IFN-γ, *Corneybacterium parvum*, L-fucose etc. are used in combination with drugs such as cyclophosphamide, cisplatin etc. aginast many types of malignancies (Malik et al., 1991). These synthetic immunomodulators have side effects such as immunosuppression, fever, fatigue, myalgias, neutropenia and liver toxicity. Herbal immunomodulators such as Rasayanas, extracts of *Withania somnifera*, *Tinospora cordifolia*, *Curcuma longa* etc. could stimulate the immune response without any side effects (Praveenkumar et al., 1999).

The results of the present study indicate that the ethanolic extract of *S. asoca* stem bark (SA) exhibited significant increase in total WBC count, realtive organ weights of spleen and thymus, suggesting the immunostimulatory effect of the extract. The increase in total leukocyte counts by the extract suggested its stimulatory effect on leukopoietic activity. The enhanced weights of thymus and spleen which represent primary and secondary lymphoid organs of the immune system indicated the immunopotentiating effect of SA.

Cell mediated immune response is involved in the effector mechanisms carried out by T-lymphocytes and their products such as lymphokines. The cell mediated immune response are very important to defence against infectious microorganisms, infections of foreign grafts, delayed type hypersensitivity reactions, detection and elimination of intracellular antigens, tumour cells etc. (Miller, 1991). Cytokines such as lymphokines are produced by the activated T-lymphocytes which attract more scavenger cells to the site of the reaction. Antigen specific cells such as $CD8^+$ cytotoxic T-lymphocytes and cytokine secreting $CD4^+T_H$ cells and antigen non-specific cells such as NK cells, macrophages, neutrophils and eosinophils are involved in cellular immune responses (Miller, 1991). Delayed type hypersensitivity (DTH) test is used to evaluate the effect on cell mediated immune response. In the present study, SA exhibited a significant ($P \leq 0.05$) dose dependent increase in DTH reaction at 24 h in mice in response to SRBC antigen compared to the control group and it is correlated to the stimulatory effect of the extract on T-cells and accessory cell types in response to cell dependent antigen like SRBC, for the development of DTH. The increase in DTH reaction in mice in response to T-cell dependent antigen revealed the stimulatory effect of SA on T-cells.

The lysosomal enzymes released during inflammation produce harmful effects and the extracellular activity of these enzymes is related to acute or chronic inflammation. The non-steroidal antiinflammatory drugs act either by inhibiting the lysosomal enzymes or by stabilizing the lysosomal membrane (Joseph et al., 2013). Since HRBC membrane is similar to lysosomal membrane which influence in the process of inflammation, the study was undertaken to check the stability of HRBC membrane by the extract. SA was subjected to check the stabilization of HRBC membrane to predict the anti-inflammatory activity. SA exhibited significant HRBC membrane stabilization effect and it can be taken as a measure of *in vitro* anti-inflammatory effect (Chippada et al., 2011) of the extract. The erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that SA may as well stabilize lysosomal membrane and its stability is important in limiting the inflammatory response. The extract may prevent the release of lysosomal constituents of activated neutrophils such as bacterial enzymes and proteases, which can cause further inflammation and its extracellular release results in tissue damage (Hossain et al., 2015).

Free radicals have very short half-life, high reactivity and damaging capacity towards macromolecules like proteins, DNA and lipids. Under physiological conditions, damage due to free radicals is countered by the natural

antioxidant system of the body. Sometimes, excessive free radical formation occurs, and the antioxidant system cannot cope with the situation leading to a condition known as oxidative stress. This results in the causation and progression of different diseases including atherosclerosis, carcinogenesis, neurodegenerative diseases, chronic inflammatory diseases, ageing and various other pathobiological effects (Irshad and Chaudhuri, 2002). Plant extracts and phyto-compounds stimulate antioxidant defense of the body without any side effects. The principle behind the wide usage of DPPH radicals to investigate the scavenging activity of some natural compounds is that, when DPPH radicals encounter a proton donating substance, it would be scavenged and the absorbance is reduced. SA exhibited significant DPPH radical scavenging activity compared to standard control. Hydroxyl radicals reacts with polyunsaturated fatty acid moieties of the cell membrane, proteins and DNA which may severely disrupt its function and lead to cell death (Valko et al., 2007). Hydroxyl radical scavenging activity can be measured by studying the competition between deoxyribose and the plant extract for hydroxyl radicals generated from the Fe^{3+}/EDTA/H_2O_2 system (Fenton's reaction). The ethanolic extract of *S. asoca* stem bark (SA) showed potent hydroxyl radical scavenging activity compared to standard ascorbic acid. Preliminary phytochemical analysis of the extract revealed the presence of carbohydrates, glycosides, saponins, steroids, terpenoids and flavonoids. It has been reported that lignin glycosides, flavonoids such as (-)-epicatechin, β-sitosterol glucoside etc. were isolated from the dried bark of *S. asoca* (Dhawan et al., 1977). The high phenol and flavonoid compounds present in the plant extract can act as strong proton donors and this is attributed to the potent free radical scavenging activity of SA.

Conclusion

In conclusion, immunomodulatory and free radical scavenging effects of ethanolic extract of *S. asoca* stem bark extract can be attributed to the presence of flavonoids and phenolic compounds present in it and the findings substantiates various traditional medicinal uses of the plant. Further studies are in progress in our laboratory to explore its active compounds and detailed mechanism of action.

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Table 1:
Effect of ethanolic extract of *Saraca asoca* stem bark (SA) on the relative organ weights (g) of Swiss albino mice

Groups	Kidney	Liver	Spleen	Thymus
Control	1.30 ± 0.07	4.12 ± 0.09	0.34 ± 0.04	0.16 ± 0.01
SA (25 mg/kg)	1.29 ± 0.08	4.14 ± 0.10	0.35 ± 0.05	0.15 ± 0.01
SA (50 mg/kg)	1.30 ± 0.08	4.17 ± 0.11	0.35 ± 0.04	0.16 ± 0.02
SA (100 mg/kg)	1.31 ± 0.09	4.18 ± 0.11	0.36 ± 0.06	0.17 ± 0.02
SA (200 mg/kg)	1.31 ± 0.09	4.20 ± 0.15	0.49 ± 0.09**	0.25 ± 0.05**

Values are the mean ± SD, n=6 in each group, Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison test. **Significance $P \leq 0.05$ compared to the control.

Table 2:
Effect of ethanolic extract of *Saraca asoca* stem bark (SA) on haematological parameters of Swiss albino mice

Groups	WBC ($\times 10^3$/mm^3)	RBC ($\times 10^6$/mm^3)	Hb (g/dl)
Control	4.35 ± 1.0	8.91 ± 0.6	12.7 ± 0.5
SA (25 mg/kg)	4.50 ± 1.2	8.92 ± 0.8	12.9 ± 0.6
SA (50 mg/kg)	4.60 ± 1.4	8.95 ± 1.2	13.2 ± 0.9
SA (100 mg/kg)	4.85 ± 1.5	8.96 ± 1.3	13.6 ± 1.2
SA (200 mg/kg)	6.25 ± 2.8**	9.01 ± 1.5	13.8 ± 1.5

Values are the mean ± SD, n=6 in each group, Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison test. **Significance $P \leq 0.05$ compared to the control.

Table 3:
Effect of ethanolic extract of *Saraca asoca* stem bark on DTH response (*in vivo*) using SRBCs as antigen in Swiss albino mice

Groups	DTH response (mm^3) 24 h	DTH response (mm^3) 48 h
Control (0.5% Tween-80)	0.13 ± 0.010	0.08 ± 0.010
SA (25 mg/kg)	0.33 ± 0.007	0.25 ± 0.020
SA (50 mg/kg)	0.44 ± 0.020	0.33 ± 0.020

SA (100 mg/kg)	0.59 ± 0.010**	0.48 ± 0.010**
SA (200 mg/kg)	0.82 ± 0.010**	0.66 ± 0.020**

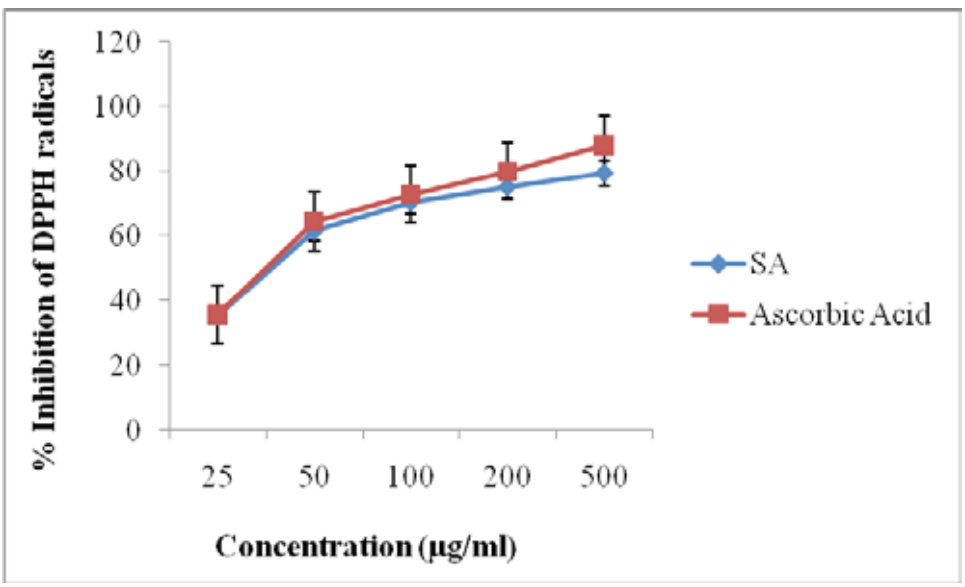
Values are the mean ± SD, n=6 in each group, Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison test. **Significance $P \leq 0.05$ compared to the control.

Table 4:
Effect of ethanolic extract of *Saraca asoca* stem bark (SA) on human red blood cell membrane (HRBC) stability *in vitro*

Groups	O. D at 560 nm	Protection of membrane stability (%)
Control (Distilled water)	0.140	------
SA (50 µg/ml)	0.075	46.43
SA (75 µg/ml)	0.032**	77.14**
SA (100 µg/ml)	0.028**	80.00**
SA (125 µg/ml)	0.023**	83.57**
SA (150 µg/ml)	0.020**	85.71**
SA (175 µg/ml)	0.019**	86.43**
SA (200 µg/ml)	0.014**	90.00**

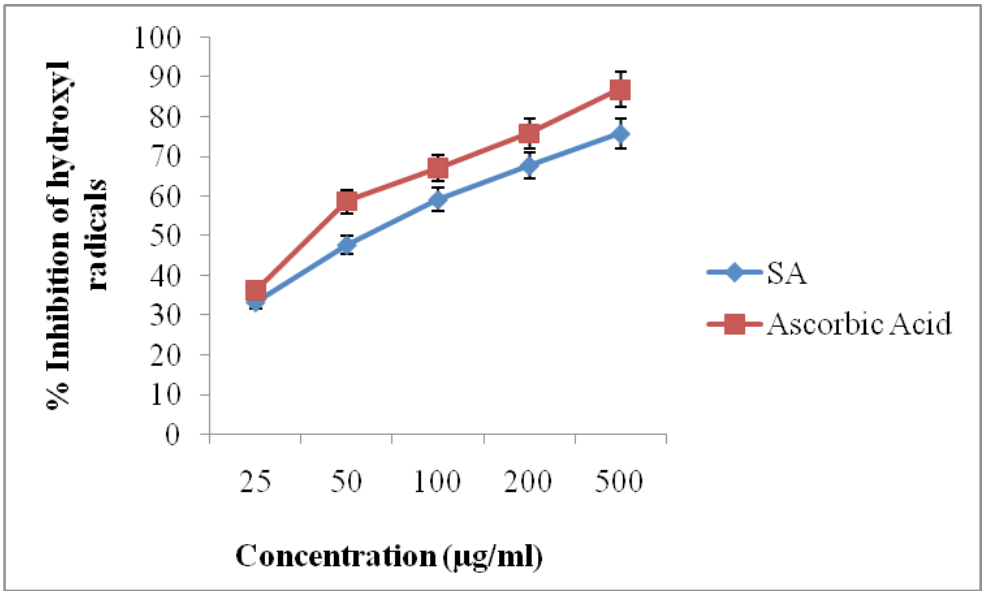
Values are the mean ± SD, n=6 in each group, Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison test. **Significance $P \leq 0.05$ compared to the control.

Figure 1:
The free radical scavenging effect of ethanolic extract of *Saraca asoca* stem bark (SA) against DPPH (1,1-diphenyl-2-picryl hydrazyl) radicals *in vitro*.



Values are the mean ± SD, n=6 in each group, Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison test. **Significance $P \leq 0.05$ compared to standard ascorbic acid control.

Figure 2:
The free radical scavenging effect of ethanolic extract of *Saraca asoca* stem bark (SA) against hydroxyl radicals *in vitro*.



Values are the mean ± SD, n=6 in each group, Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison test. **Significance $P \leq 0.05$ compared to standard ascorbic acid control.