Original article

Oxidative free radicals scavenging activity \textit{(in vitro and in vivo assay)} of standardized fractions from the seeds of \textit{Argyreia speciosa} (Ghav-patta) a traditional Indian medicine

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\textbf{A B S T R A C T}

Traditional pertinence: \textit{Argyreia speciosa} Sweet (Linn.), belongs to the family convolvulaceae, a traditional Indian medicinal herb, has been used to treat acute/chronic ulcers, gonorrhea, rheumatoid arthritis and several nervous disorders having a long history.

\textit{Aim of the study:} A broad spectrum approach of this work was to find out the antioxidant activity of \textit{Argyreia speciosa} seeds, \textit{in vitro} and \textit{in vivo} antioxidant assay were performed.

\textit{Material and methods:} Total phenolic content (TPC), reducing power (RP), antioxidant activity (AOA), $O_2^-$ (superoxide anion), DPPH (1,1-diphenyl-2-picrylhydrazyl) and $OH^-$ (hydroxyl) radicals scavenging activities, GSH (glutathione), CAT (catalase), SOD (superoxide dismutase), and LPO (lipid peroxidase) are the major parameters which were studied for determining \textit{in vitro} and \textit{in vivo} antioxidant property of seed extract and their six fractions obtained from \textit{A. speciosa}. Carbon tetrachloride (CCl$_4$) induced rat model was used to determine in vivo antioxidant assay of extract and its fractions.

\textit{Results:} Butanol fraction (AS-BF) showed strong antioxidant property and protected oxidative DNA damage. AS-BF was found best as compared to all other fraction for determining antioxidant property of seeds extract & their six fractions obtained from \textit{A. speciosa}. Carbon tetrachloride (CCl$_4$) induced rat model was used to determine in vivo antioxidant assay of extract and its fractions.

\textit{Conclusion:} From these results, it was proved that \textit{A. speciosa} seeds prevent tissue damage due to oxidative stress with strong antioxidant activity.

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1. \textbf{Introduction}

In the case of human body, oxidative free radicals possess a very important role for various biological activities, such as the intercellular killing of microbes by phagocytic cells like macrophages and granulocytes (Bhagat et al., 2016). They are involved in a certain cell signaling process known as redox signaling (Pacher et al., 2007). From the latest researches, it is found that oxygen-centered ROS which is abbreviated as reactive oxygen species e.g. superoxide and hydroxyl radical play a very important role in the case of cell signaling (Singh et al., 2017b). However, because of their reactive nature, they are involved in unwanted side reactions causes cell damage. Excess of these ROS resulting in cell damage and cell death. Due to these reasons they pony up maximum diseases e.g. diabetes, cancer, cardiac damage and immunological disease. The body has strong mechanisms to minimize cellular damage by ROS (Vlasova, 2018). In addition, antioxidants obtained from different sources play a very important role in the defense mechanisms of ROS (Sarma et al., 2010). Plants are a rich source
fraction (AS-AF) were obtained. Solvents from these fractions were evaporated and dried completely. The dried extracts were used for the preliminary screening, in vitro and in vitro antioxidant activity.

2.4. Polyphenolic content determination (TPC)

Phenolic contents of A. speciosa seeds extract were calculated with slight modification (Ragazzi and Veronese, 1973). 10 mg of seeds extract was mixed with 1.0 ml of Folin’s reagent (1 N) and 2.0 ml of Na₂CO₃ (20%) was added subsequently. The spectrum of samples was measured at 727 nm and phenolic contents were expressed as mg/g equivalence of Gallic acid (GAE).

2.5. Flavonoid content determination (TFC)

The colorimetric assay method was used to calculate total flavonoid content with some modification (Oyaizu, 1986). UV/visible absorbance of seeds extract was determined at 510 nm. Quercetin (0 to 500 mg/L, Merck, Mumbai, India) was taken as a standard and flavonoid content was determined as (Quercetin)/g of the seeds extract.

2.6. In vitro antioxidant activity

Beta-carotene and linoleic acid test was used for determination of antioxidant activity with some modification in the method explained by Emmons and Peterson (1999) and it was represented as percentage inhibition (PI%). Ferric reduction antioxidant assay method was used to measure the reducing capacity (RP) (Apati et al., 2003). 1,1-diphenyl-2-picrylhydrazyl solution (DPPH)×10⁻⁵ M in methanol) was used to determine free radicals by some modification in method given by Ven and Pin-Der (1994), efficiency concentration (EC₅₀), inhibitory concentration (IC₅₀), anti-radical power (ARP) and superoxide ion free radical scavenging activity assay (FRSA) (Kroyer, 2004; Nishikimi et al., 1972). Hydroxyl radical scavenging activity was determined by Halliwell and Gutteridge (2015). Chelation capacity of ferrous ion was determined by Decker and Welch (1990). Lipid peroxide formation from lipid-rich rat liver homogenates and its inhibition were determined by TBARS abbreviated as Thiobarbituric acid-reactive species assay (Ohkawa et al., 1979).

2.7. Chromatographical analysis

For purification and determination of HPLC analysis silica gel which is pre-coated-60 F254 plate (Merck, Mumbai, India) having a thickness of 0.2 mm was used. Linomat IV (CAMAG) was used for sample application and plates were scanned under the scanner (CAMAG-3).

2.8. Nicking protective effect of DNA induced by OH free radical

Supercoiled pBR322 DNA was used for determining the protective effect of seeds extract by DNA nicking. Lee method with some modification was used for determining DNA nicking (Lee et al., 2002), quercetin (50 μM) and catalase (5 units) were used as reference one.

2.9. In vivo antioxidant activity

Wister rats were used to determine in vivo antioxidant activity. Rats were divided into 5 respective groups (n = 6 in each group). First group: Normal control group (1% CMC, p.o.); Second group: Negative control group (CCl₄ induced group, CCl₄: liquid paraffin (1:1; 2 ml/kg body weight/day, s.c.); Third group: Treatment group (CCl₄ + AS-BE 50 mg/kg body weight/day, p.o.); Fourth group:
(CCl₄ + AS-BF 100 mg/kg body weight/day, p.o.); Fifth group: Reference group (CCl₄ + Vitamin E, 50 mg/kg body weight/day, p.o.). Rats were sacrificed after 24 h of the last dose of CCl₄. Supernatants were used for determination of biochemical parameters. Malondialdehyde (MDA) level were estimated by the method of Ohkawa et al. (1979). CAT, SOD, and GSH most important parameters of in vivo antioxidant activity were estimated by the method explained by Aebi (1974), Kakkar et al. (1984), and Ellman (1959).

2.10. Statistical determination

One-way ANOVA was used for statistical determination and it was completely assayed by Newman Keuls Multiple Comparison followed by Scheffe’s test with software Graph pad Prism 7. Studies were explained in the form of mean ± SEM to measure the difference in each group and \( P < 0.05 \).

3. Results

TPC ranged from 27.7–117.2 mg/g in all six fractions of A. speciosa seeds extract. AS-BF showed maximum phenolic content and it has decreased respectively in this order AS-BF > AS-EF > AS-CE > AS-DF > AS-CF > AS-AF (Fig. 1.). The TFC in various fractions were varied from 3.367 to 25.54 mg/g (quercetin maximum). The antioxidant activity of all fractions of seed extract ranged from 21.43%–96.78%. Maximum antioxidant activity was found in AS-BF and range went down in respective pattern AS-BF > AS-EF > AS-CE > AS-DF > AS-CF > AS-AF, in AS-BF antioxidant activity percentage was found to be 52.13 ± 2.01% (Fig. 1.). Reducing power (RP) level ranged from 0.56 to 2.21 (Fig. 2A.). Maximum reducing power was shown in AS-BF, it was determined by the low value of ASE/ml (0.56) and other fractions having RP value respectively: AS-BF > AS-EF > AS-CE > AS-DF > AS-CF > AS-AF.

Inhibitory concentration ranged from 0.032 to 1.20 mg/ml in all six fractions of A. speciosa seeds and effective concentration was 1.24–52.21 mg/dpph and level of ARP was found as 1.87–79.68. Butanol fraction showed maximum DPPH scavenging activity and it was proved by the lower level of IC₅₀ and EC₅₀ and higher ARP (Fig. 2A.). Scavenging activity of all six fractions was expressed in the given order: AS-BF > AS-EF > AS-CE > AS-DF > AS-CF > AS-AF. Superoxide anion radical activity in AS-EF (0.17 mg/ml) and AS-BF (0.09 mg/ml) was determined very potent and in case of AS-DF, AS-CE, AS-AF and quercetin values were ranges as 0.72, 1.21, 1.83 and 0.03 mg/ml (Fig. 2B). AS-BF showed the maximum value of non-site specific inhibition of hydroxyl radicals. The result was proved by the low value of IC₅₀ (0.16 mg/ml) and data were analyzed for rest fractions as follows: AS-BF > AS-EF > AS-CE > AS-DF > AS-CF > AS-AF. These conditions proved that AS-BF work as metal chelates. Alcoholic fraction has minimum antioxidant property due to least IC₅₀ 1.21 mg/ml. Metal chelation ranged for all fraction as: AS-BF > AS-EF > AS-CE > AS-DF > AS-CF > AS-AF (Fig. 3). Chelating capacity was expressed as the value of IC₅₀ and it was varied from 0.09 to 2.13 mg/ml (Fig. 3). MDA generation was inhibited by AS-BF in its concentration-dependent way and its IC₅₀ value were decreased in this way: AS-BF (0.061 mg/ml) > AS-EF (0.09) > AS-CE (0.21 mg/ ml) > AS-DF (0.29 mg/ml) > AS-CF (0.52 mg/ml) > AS-AF (0.98 mg/ml).
From these data it was showed that butanol fraction has the best antioxidant activity in in vitro study as compared with other fractions. Henceforth, AS-BF was further used to determine protective oxidative damage and in vivo antioxidant property determination.

HPLC analysis was used for determining the presence of polyphenols: Gallic acid (556.7 μg/gm), kaempferol (497.6 μg/g), ferulic acid (128.2 μg/g), ellagic acid (199.1 μg/g), rutin (190 μg/g), quercetin (804 μg/g) and chlorogenic acid (482.5 μg/g) in AS-BF. Presence of these bioactive compounds was further proved by IR and MS analysis of seed extracts.

AS-BF (Lane 4 to 10) showed concentration-dependent DNA nicking protection it was found in the range 5–25 μg/ml. Lowest concentration and protection was at Lane 4 and the highest level of protection of DNA was at 10 μg/ml. Results were compared with 5U of Catalase at Lane 3 and standard quercetin 50 μM - Lane 11 (Fig. 4). CCl4 intoxication leads to an increase in the concentration of LPO. Its significant coefficient value is<0.001, there is quite a reduction in GSH value (p < 0.001), CAT level (p < 0.001) and SOD content (p value is < 0.001) in different organs especially in kidney and liver as compared to healthy one (Figs. 5 and 6). AS-BF treatment at dose 50 and 100 mg/kg showed strong effect against CCl4 intoxication and also inhibited increased content of LPO, increased SOD, GSH and CAT levels in CCl4 intoxicated rats with a significant coefficient (p < 0.001) (Figs. 5 and 6).

4. Discussion

A. speciosa seeds and its six fractions were studied for their in vitro and in vivo antioxidant activity, at the same time their oxidative DNA nicking study was also performed. As compared to all five fractions of seed extract, butanol fraction showed the highest level of phenolic and flavonoid content. This was the one of the most important reasons for its strength in in vitro and in vivo antioxidant activity. Antioxidant phenolic acids were present in seed extracts and their results were proved by HPLC analysis.

Linoleic acid and beta-carotene were used to determine oxidative free radicals scavenging activity of A. speciosa. As β-carotene bleaching capacity was hindered by free radical neutralization showed the presence of different antioxidants (Juntachote and Berghofer, 2005). AS-BF represents strong antioxidant activity due to a decrease in β-carotene bleaching. Reducing power of A. speciosa seeds was determinant to its ability of electron transfer which is the strong determination of antioxidant activity (Chung et al., 2002). DPPH assay was determined at 517 nm and their scavenging property was determined by discoloration of DPPH. The
Castillo, 2008). Hydroxyl free radicals initiate a chain type of reaction and have strong antioxidant activity (Benavente-García and researches has proven that polyphenols are superoxide scavengers activity of AS-BF fraction. Both site-specific and non-site specific deoxyribose assay was used for the confirmation of the antioxidant action of AS-BF fraction. Both site-specific and non-site specific degeneration of free radicals were studied.

Antioxidant activity of A. speciosa seeds was further proved with the help of chelation capacity of ferrous ion and they are in vitro lipid peroxide formation from rat liver homogenate. Transition metals were well stabilized by chelating agents and follow the catalytic type of reaction (Moore and Yu, 2008). Lipid peroxidation assay was determined by MDA formation. This study represented that butanol fraction inhibited the level of MDA which was due to the absence of ferryl/perferryl complex (Govindarajan et al., 2003). From results, it was observed that AS-BF showed free radical scavenging property at different concentration were observed at in vitro study, it was also proven that AS-BF prevents tissue damage which was due to oxidative stress. These findings promote the preventive effect of A. speciosa seeds and explain the traditional use of it in every day for healthy life.

Declaration of Competing Interest

None.

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