INTRODUCTION

Free radicals are generated within the cells through the metabolic process and they are important for certain cellular responses at low or moderate concentrations. The increase in the concentration of these free radicals can happen when there is an imbalance between their overproduction and the deficiency in free radicals scavenging enzymes and molecules [1]. Exposure to external stress (toxins, radiation, carcinogens) can also lead to the accumulation of free radicals [2-5]. Excessive concentrations of these radicals lead to a situation called as "oxidative stress" and "nitrosative stress" whereby these radicals interact with bio-molecules such as DNA, proteins and lipids and inhibit their normal functions [1]. Oxidative stress and nitrosative stress thus leads to the implications of various diseases such as cancer, diabetes, cardiovascular diseases, neurodegenerative disorders etc. [6-10]. To counter the effects of free radicals damage, it is required to bring the free radicals within normal levels. Several studies have shown the positive effects of antioxidant on ameliorating diseases like cancer, diabetes, alzheimer etc. [11, 12]. Synthetic antioxidants such as butylated hydroxytoluene and butylated hydroxyanisole have been commonly used to maintain the quality of ready-to-eat food products, natural antioxidants, however, were found to have higher antioxidant activity when compared with the synthetic ones [13]. Plant-derived antioxidants are of interest due to their potent preventive, as well as therapeutic actions [14]. In this study, the antioxidant activity of Aristolochia tagala which have been reported to have various biological properties was evaluated and LC/MS analysis of compounds present in the fraction with highest antioxidant activity was carried out.

MATERIALS AND METHODS

Preparation and fractionation of Aristolochia tagala aqueous-methanol extract

Crude aqueous-methanol extract (4:1 v/v) methanol:water (100 g in 250 mL) was prepared from powdered dried roots of A. tagala. The crude extract was designated as ATC. Fractionation of ATC was carried out by open column chromatography in silica gel (100-200 mesh). The extract were eluted out with gradients of CHCl3:CH3OH, 100 ml of 95:5 and 50 ml of 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90 and 0:10. Various fractions of 15 ml each were collected in a clean test tube. Ultraviolet (UV)-visible spectra of individual fractions were recorded and the fractions were combined based on their Amax [15]. They were then dried to completely remove the solvents and varying concentration of ATC and fractions were prepared in methanol and assayed for their antioxidant activity.

Antioxidant properties of ATC and fractions

The antioxidant properties of ATC and fractions were assayed by their ability to scavenge free radicals like superoxide, nitric oxide and ABTS radical cation and the identification of compounds was carried out by LC/MS analysis.

RESULTS

Fraction I of Aristolochia tagala showed the highest free radical scavenging activity and compounds responsible for its activity were identified as magnoflorine, apigenin dimethyl ether, aristolone, and N-trans-feruloyldopamine.

Conclusion

The free radical scavenging property of the compounds present in Aristolochia tagala may be one mechanism that contributed to medicinal property exhibited by this plant.

Keywords: Aristolochia tagala, Oxidative stress, Antioxidant activity
ABTS** solution was added to 100 μl of samples at varying concentrations in a 96 well plate. The absorbance was measured immediately after 5 min at 734 nm.

**Mass spectrometric analysis of the fraction of *A. tagala***

LC/MS was carried out in XEVO-TQD coupled with Waters Alliance 2695 HPLC system (Waters, USA). Fraction I (1 mg/ml) was prepared in methanol and filtered through a 0.22-μm PVDF membrane. C18 SUNFIRE column (250 mm × 4.6 mm, 5 μm) (Waters, USA) was used for LC analyses, the column temperature was set at 30 °C. 20 μl of sample was injected automatically by Waters Alliance 2695 autosampler. The mobile phase consists of acetonitrile (A) and 5 mmol ammonium acetate buffer (B). The gradient programme was 95 % B for 0 -6 min, 70 % B for 6-12 min, 40 % for 12-16 min, 40 % for 16-20 min, 20 % for 20-24, 20 % for 24-26 min, and 95 % for 26-30 min. Electron Spray Ionisation (ESI) analysis was carried out in XEVO-TQD. The desolvation and cone gas flow were at 950 L/h and 30 L/h respectively, capillary voltage 3500V, cone voltage 30V, source temperature 125 °C, desolvation temperature 350 °C. Collision energy for MS/MS analysis was 10 eV. The range for the full ESI scan was set from 150 to 1000 in m/z. Data acquisition and processing were carried out using MassLynx V4.1 SCN 714 software [19].

**Data and statistical analysis**

Statistical analysis was performed by GraphPad Prism 5 Software (GraphPad Software, Inc., USA) using one-way anova followed by Tukey’s multiple comparisons test. Data are presented as mean±standard deviation. Significance was set at P<0.05.

**RESULTS**

Fractionation of the aqueous-methanol extract of roots of *Aristolochia tagala* yielded thirty-eight fractions which were pooled into four fractions (I-IV) as previously reported [15]. To assess the free radical scavenging potential of ATC and the fractions, the reactivity toward nitric oxide radical (NO.), superoxide radical (O2.–) and free radical ABTS** was measured. The absorbance was converted into an AA% which was calculated using the formula AA% = [Abscontrol - (AbsSample – AbsBlank)]/Abscontrol × 100]. The results are expressed as IC50 value which is the concentration of extract that was able to scavenge 50 % of the free radical. A lower IC50 value corresponds to a high antioxidant property and *vice versa*.

**Table 1: IC50 (µg/µl) of crude extract or fractions of *A. tagala***

|   | NO– | O2– | ABTS** |
|---|-----|-----|--------|
| F I | 102±0.189*** | 122±1.599*** | 119±0.312*** |
| F II | 112±0.222*** | 162±0.150*** | 1240±0.031*** |
| F III | 172±0.022*** | 135±0.0454*** | 1170±0.143*** |
| F IV | 200±0.761*** | 570±0.515*** | 1650±0.272*** |

The fractions of *A. tagala* were able to scavenge free radical generated more readily than the crude extract (ATC). The scavenging effect was more prominent for NO radical and ABTS. Fraction I (F I) showed the highest scavenging activity with an IC50 value of 102 µg/ml, 152 µg/ml, 850 µg/ml for NO, O2– and ABTS respectively (table 1).

The total ion chromatogram obtained from LC/MS analysis of F I showed the presence of several compounds (fig 1). The compound with highest relative abundance was at a retention time (RT) of 24.05 min in the ESI+ mode and 23.85 min in the ESI-mode. The compound at RT of 24.05 min showed formation of [M+H]+ at m/z 343.4, adduct ion formation [M+Na]+ at m/z 365.4 and dimer formation [2M+H]+ at 687.7 and [2M+Na]+ at m/z 707.7. The compound at RT 23.85 min showed [M+H]+ at m/z 325.3 (table 2). These compounds were tentatively identified as magnoflorine and aristolide C respectively based on literature reports of compounds present in *A. tagala* and related species of the same genus and compared to compounds listed in databases Knapsack (http://kanaya.naist.jp) and CHEMnet BASE-Dictionary of Natural Products (http://dnp.chemnetbase.com).

**Fig. 1: Total ion chromatogram (TIC) of fraction I of *Aristolochia tagala* (a) ESI+ mode and (b) ESI-mode**

**Table 2: Compounds present in Fraction I of *Aristolochia tagala***

| S. No. | RT | %RA | m/z [M+H]+ | m/z [M-H] | Compound name |
|-------|----|-----|------------|-----------|--------------|
| 1     | 12.35 | 30 | 330.5 |          | N-Trans-Feruloyldopamine |
| 2     | 12.54 | 40 | 239.3 |          | Aromadendrene 4β,10β-diol |
| 3     | 14.17 | 75 | 372.5 |          | Aristolochic acid IV |
| 4     | 14.69 | 92 | 326.2 |          | Aristolochic acid la |
| 5     | 20.94 | 55 | 372.5 |          | 7 methoxy aristolochic acid a |
| 6     | 23.85 | 100 | 219.3 |          | 3-Oxoisohwarane |
| 7     | 23.85 | 100 | 325.3 |          | Aristolide C |
| 8     | 24.05 | 100 | 343.4 |          | Magnoflorine |
| 9     | 24.18 | 70 | 339.4 |          | Aristofolin C |
| 10    | 25.48 | 60 | 297.3 |          | Apigenin dimethyl ether |
| 11    | 26.12 | 90 | 159 |          | Aristolone |
DISCUSSION
Natural antioxidants are present in a wide variety of food and medicinal plants and they have been reported to have a wide range of biochemical activities, including inhibition of ROS generation, direct or indirect scavenging of free radicals, and alteration of intracellular redox potential [20, 21]. The in vitro antioxidant activities studies can be used to confer the antioxidant potential of medicinal plants which in turn can give an insight into the role and mechanism of plants in alleviating pathophysiological diseases caused by oxidative stress. Different groups of phytochemicals contribute to the antioxidant activity of plants. Phenolic antioxidants have shown to have excellent antioxidant activity, while other groups of phytochemicals like terpenoids, alkaloids have shown to have moderate antioxidant activity [20]. The antioxidant activity exhibited by Fraction I of A. tagala was due to the present of compounds like magnoflorine which was reported to have antioxidant activity both in vitro and in vivo system and was also reported to have anti-inflammatory activity [22-24], apigenin dimethyl ether; reported to have potential antidiabetic and anti-obesity properties [23, 25], Aristolone, N-Trans-Feruloyldopamine previously reported to have antioxidant activity [26, 27]. Aristolochia tagala have been reported to exhibit potential anti-cancer activity, anti-inflammatory activity, antimicrobial activity [15, 28-30]. The medicinal property exhibited by A. tagala may be contributed by the antioxidants present in this plant which may have helped in reducing the oxidative stress through the scavenging of free radicals generated by exogenous stimuli under different experimental conditions. A. tagala therefore is a good source for extraction of these compounds which shows antioxidant property as well as pathophysiological activity.

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AUTHORS CONTRIBUTIONS
All the author have contributed equally

CONFLICTS OF INTERESTS
The authors have no conflict of interest to declare

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