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Research Article

ANTI-INFLAMMATORY AND ANTIOXIDANT POTENTIAL OF *ABUTILON INDICUM*: *IN VITRO* STUDY

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ABSTRACT

The aims of this study were to screen the phytochemical compounds and to evaluate *in vitro* antioxidant and anti-inflammatory activities in the ethanolic extract of *Abutilon indicum* (EtAI) leaves. *Abutilon indicum* leaves were subjected under phytochemical screening using five different solvents (distilled water, ethanol, acetone, petroleum ether and ethyl acetate) with different polarities. The flavonoid and phenolic content of EtAI was determined using standard biochemical methods. EtAI was assessed for ABTS radical scavenging, hydroxyl radical scavenging, reducing power and nitric oxide radical scavenging assays. We also analysed the anti-inflammatory potential of EtAI using the inhibition of bovine serum albumin denaturation assay. Phytochemical analyses revealed the presence of alkaloids, phenols, flavonoids, proteins, carbohydrates, triterpenoids and saponins in EtAI. The total phenolic content of EtAI was 5.82 mg CE/g of extract. The total flavonoid content of EtAI was 7.91 mg RE/g of extract. EtAI exhibited strong scavenging activity in a concentration-dependent manner in all the scavenging assays. The EC_{50} of EtAI on ABTS, reducing power, hydroxyl and nitric oxide radical were found to be 2.65, 1.94, 3.66 and 0.62 mg/ml, respectively. It also showed excellent anti-inflammatory activity (IC_{50} = 227 µg /ml). Our findings provide evidence that the ethanolic extract of *Abutilon indicum* (EtAI) leaves possess anti-inflammatory activity and is a potential source of natural antioxidants.

Key Words: *Abutilon indicum*, Anti-inflammation, Antioxidants, Free radicals, FT-IR, Phytochemicals

INTRODUCTION

Living cells may produce free radicals and other stable reactive oxygen species (ROS) as by-products of various physiological processes. These ROS can cause cellular damage to biological macromolecules and eventually leading to many chronic and degenerative diseases in humans[1]. Certain types of inflammatory diseases are mediated by ROS. The sources of these oxidizing agents are the neutrophils, eosinophils, monocytes and macrophages which invade tissues. Inflammation is a complex process which involves many occurrences includes enzyme activation, release of chemical mediators, protein denaturation, tissue repair and membrane alterations[2].

Inflammation is a healing process in which organism remove injurious stimuli. If the inflammation is not treated, it leads to the development of other diseases like atherosclerosis and rheumatoid arthritis[3]. Because of the side effects of currently available drugs in the market such as non-steroidal anti-inflammatory drugs (NSAIDS) and opioids, a search for alternatives becomes much essential now[4]. Plant-derived bioactive compounds have recently become of much interest due to their versatile applications[5]. The plants which are used in traditional systems of medicine are the richest bio-resource of drugs for modern medicines[6]. China and India have a well-established herbal medicines industry and they are investing much in medicinal plants research[7].

In this regard one such plant *Abutilon indicum* (*A. indicum)* belonging to the species *Abutilon* is known for its medicinal uses in traditional system of medicine. It is used in Ayurveda, folk medicines, siddha, unani and Tibetian medicine system. In Hindi, it is called as “thuththi” or “atibala” or “kanghi”. It is also called as “country mallow” and it is a perennial plant upto 3m height. The whole plant as well as specific part such as flower, leaves and fruit is also used to treat various health problems[8]. It is a native plant of south Asia. It is extensively grown in Bangladesh, India, Pakistan and Srilanka. This plant is used as a traditional medicine as a laxative, emollient, analgesic, anti-diabetic, anti-inflammatory, antimicrobial activity and also to treat urinary disease, piles, jaundice etc.,[9].

Pharmacological studies reveal that *A. indicum* has some medicinal properties such as anti-diabetes, anti-microbial effect, anti-inflammatory, anti-cancer, anti-atherosclerotic effect and anti-hepatoprotective activity[10]. However, little is known about the anti-inflammatory effect of leaf extracts of *A. indicum*. Therefore, in the present study, we analysed the presence of phytochemicals in five different extracts of *A. indicum* leaves and also studied its antioxidant and anti-inflammatory effects in ethanol extract.

MATERIALS AND METHODS

Chemicals and reagents

ABTS (2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonicacid), DMSO, phosphate buffered saline (PBS) and other chemicals were obtained from Sigma-Aldrich (Bangalore, India). Catechin, ascorbic acid, Acetyl salicylic acid, BSA (Bovine serum

albumin), rutin and gallic acid were obtained from Himedia (Mumbai, India). All other chemicals are of analytical grade.

Plant material

The fresh and healthy leaves of *Abutilon indicum* were collected in the month of December 2018 from the areas in and around Coimbatore, Tamil Nadu**.**

Preparation of extracts

The leaves of *A. indicum* were shade dried, powered and extracted using five different solvents such as distilled water (AqAI), ethanol (EtAI), acetone (AcAI), petroleum ether (PeAI) and ethyl acetate (EaAI) in a Soxhlet apparatus. The solvent was evaporated using a rotary evaporator under reduced pressure at 45°C and the obtained dried extracts were stored at −20°C for further use.

Qualitative analysis of phytochemicals

For preliminary phytochemical screening, standard assays were performed in different extracts of *Abutilon indicum*. Phytoconstituents such as carbohydrates, proteins, phenols, glycosides, triterpenoids, saponins, alkaloids, flavonoids and steroids were examined using different biochemical tests[11].

FT-IR spectral analysis

The FT-IR spectrum of EtAI was recorded with a FT- IR spectrophotometer (IR-affinity 1, Sigma, Japan) using KBr pellet method[12]. The dried extract was grounded with KBr powder and then pressed into 1mm pellets for FT-IR measurement from 4000 to 400cm^{-1}.

Estimation of total phenol

The total phenol in EtAI was measured using Singleton and Rossi method[13]. The extract (1.0 ml) was mixed with 1.0 ml of Folin phenol reagent and after 3 min, 1.0 ml of saturated sodium carbonate (35%) was added to the mixture and made up to 10 ml with deionised water. The mixture was kept for 90 min at room temperature in the dark and measures the absorbance at 725 nm against the blank. Catechin was used as the standard drug. The total phenol content is expressed as milligrams of catechin equivalents (CE) per gram of extract.

Estimation of total flavonoid

Total flavonoid content in EtAI was determined according to the method of Jia *et al*[14]. The extract (0.25 ml) was diluted with distilled water (1.25 ml). Then 5% sodium nitrite (75 µl) was added into it. After 6 min, 10% aluminium chloride (150 µl) were added and mixed. 0.5 ml of 1 M sodium hydroxide was added after 5 min and measures the absorbance at 510 nm against a blank. Rutin was used as the standard drug. The total flavonoid content is expressed as milligrams of rutin equivalents (RE) per gram of extract.

Total antioxidant capacity by Phosphomolybdenum assay

The total antioxidant capacity of the sample was measured using phosphomolybdenum method[15]. An aliquot of 0.1 ml of EtAI was mixed with 1.0 ml of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated at 95°C for 90 min. Then the tubes were cooled and measure the absorbance at 695 nm against a blank. Ascorbic acid was used as a standard drug and total antioxidant capacity was expressed as milligrams of ascorbic acid equivalents (AAE) per gram of extract.

ABTS radical cation scavenging activity

The ABTS radical scavenging assay was evaluated according to the method of Re *et al*[16]. The ABTS radicals were produced by the reaction between 7 mM ABTS and 2.45 mM potassium persulfate for 12 h (stored in dark) at room temperature. Prior to use, the solution was diluted with ethanol to get an absorbance of 0.700±0.025 at 734 nm. Then 10 µl of extract at different concentration (1, 2, 3, 4 and 5 mg/ml) was mixed with 1.0 ml of ABTS working standard in a microcuvette. After 6 min, the decrease in absorbance was measured at spectrophotometer. The percentage inhibition was calculated according to the formula: $[(A_{control} - A_{test}) / A_{control}] \times 100$, where $A_{control}$ was the absorbance of the control, and A_{test} was the absorbance of the sample.

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity of EtAI was determined using the method of Smirnoff and Cumbes[17]. To 1.0 ml of varied concentrations of the extract (1.5, 3, 4.5, 6 and 7.5 mg/ml), added 1.0 ml of 1.5 mM $FeSO_4$, 0.7 ml of 6 mM hydrogen peroxide, 0.3 ml of 20 mM sodium salicylate and incubated it for 1 h at 37°C. The absorbance of the hydroxylated salicylate complex was measured using spectrophotometer at 562 nm. The hydroxyl radical scavenging activity was calculated as follows: $[1- (A_{test1} - A_{test2}) / A_{control}] \times 100$, where $A_{control}$ is absorbance of the control (without extract) and A_{test1} is the absorbance of extract with sodium salicylate, A_{test2} is the absorbance without sodium salicylate.

Determination of reducing power

The reducing power of EtAI was evaluated according to the method of Oyaizu[18]. To 2.5 ml of extract at various concentrations (0.8, 1.6, 2.4, 3.2 and 4 mg/ml), added 2.5 ml of phosphate buffer and 2.5 ml of 1% potassium ferricyanide and incubated for 20 min at 50°C. Then 2.5 ml of 10% TCA was added and centrifuged the mixture for 10 min at 5000 g. To the supernatant (2.5 ml), deionised water (2.5 ml) and 0.1% ferric chloride (0.5 ml) were added, mixed well and after 10 min of incubation, measured the absorbance at 700 nm against a blank.

Nitric oxide scavenging assay

Nitric oxide (NO) scavenging activity was measured using Griess reaction[19]. EtAI (dissolved in DMSO) at various concentrations (0.5, 1, 1.5, 2 and 2.5 mg/ml) was mixed with sodium nitroprusside (5 mM) in phosphate-buffered saline and incubated for 3h at room temperature. After incubation, 500 µl of sample was diluted with 500 µl of griess reagent and measured the absorbance at 695 nm against a blank. The percentage nitrite radical scavenging activity of EtAI was calculated using the following formula: $[(A_{control} - A_{test}) / A_{control}] \times 100$, where $A_{control}$ was the absorbance of the control, and A_{test} was the absorbance of the test.

***In vitro* anti-inflammatory activity by protein (BSA) denaturation method**

Ethanolic extract of *A. indicum* (0.05 ml) at various concentrations (100, 200, 300, 400 and 500 µg/ml) and standard drug, aspirin (0.05 ml) at different concentrations (100, 200, 300, 400 and 500 µg/ml) were taken separately. Then 0.45 ml of 0.5% BSA was added to both test and standard drug solution. For test

control, 0.05 ml of distilled water was added with 0.45ml of BSA. After 20 min incubation at 37°c, the temperature of the samples was increased progressively up to 57°C for 3 min. Cool the above mixture, then added 2.5 ml of phosphate buffer and measured the absorbance at 416 nm using UV-Visible spectrophotometer[20]. The results were compared with the standard drug. The percentage inhibition of protein denaturation can be calculated as. Percentage Inhibition = 100 – [{(Absorbance of test – Absorbance of standard drug) / Absorbance of test}/ × 100].

Table 1: Preliminary phytochemical analysis of *A. indicum* extracts

S. No.	Phytochemical compounds	Extracts				
		AqAI	EtAI	AcAI	PeAI	EaAI
1.	Alkaloids	-	+	-	+	+
2.	Phenols	+	+	+	+	+
3.	Flavonoids	+	+	+	-	+
4.	Proteins	+	+	-	-	-
5.	Carbohydrates	-	+	-	-	+
6.	Glycosides	-	-	-	-	-
7.	Triterpenoids	+	+	-	+	-
8.	Steroids	+	-	-	-	-
9.	Saponins	-	+	+	+	+

'+' indicates the presence of particular compound; '-' indicates the absence of particular compound; AqAI = *A. indicum* aqueous extract; EtAI = *A. indicum* ethanol extract; AcAI = *A. indicum* acetone extract; PeAI = *A. indicum* petroleum ether extract; EaAI = *A. indicum* ethyl acetate extract.

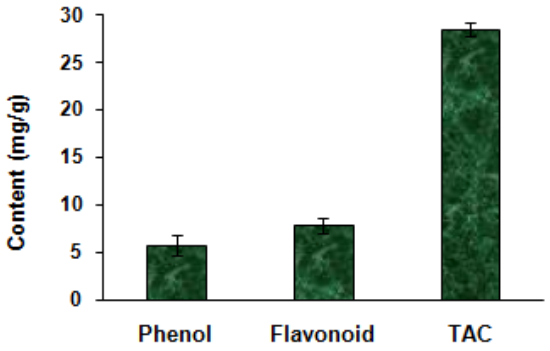

Figure 1: Total phenol (mg CE/g), flavonoid (mg RE/g) content and total antioxidant capacity (mg AAE/g) of EtAI. Each value is expressed as mean±SD (n=3). EtAI = Ethanol extract of *A. indicum*; CE = Catechin equivalents; RE = Rutin equivalents; AAE = Ascorbic acid equivalents.

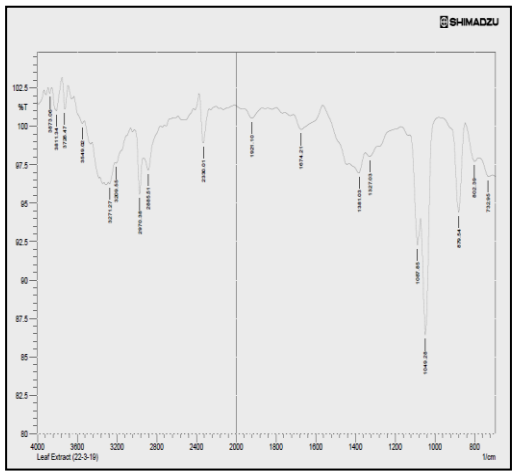

Figure 2: FT-IR of EtAI; EtAI = Ethanol extract of *A. indicum*.

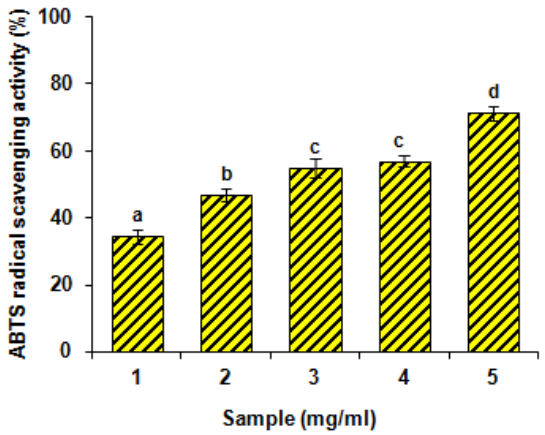

Figure 3: ABTS radical scavenging assay of EtAI. Each value is expressed as mean±SD (n=3). Different letters (a-e) indicate a significant difference between the concentrations of the same extract (p<0.05, ANOVA, DMRT); EtAI = Ethanol extract of *A. indicum*.

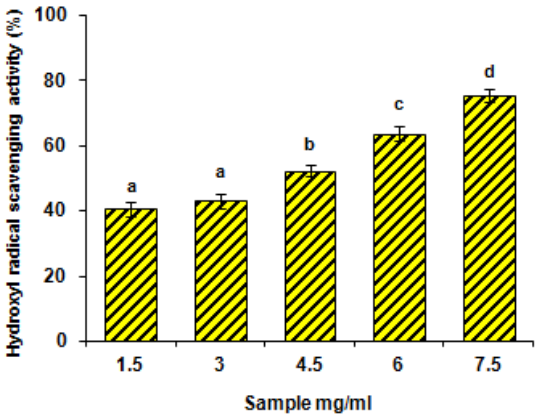

Figure 4: Hydroxyl radical scavenging assay of EtAI. Each value is expressed as mean±SD (n=3). Different letters (a-e) indicate a significant difference between the concentrations of the same extract (p<0.05, ANOVA, DMRT); EtAI = Ethanol extract of *A. indicum*.

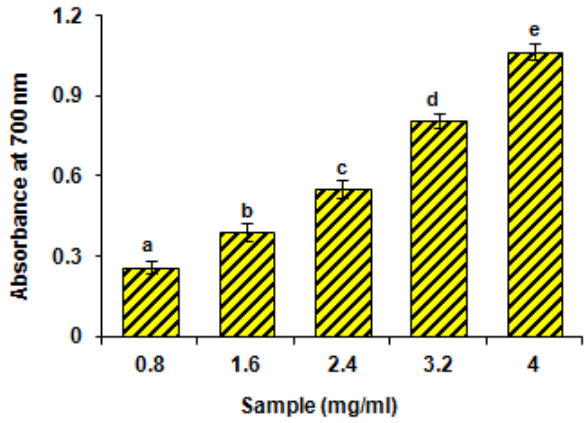

Figure 5: Reducing power assay of EtAI. Each value is expressed as mean±SD (n=3). Different letters (a-e) indicate a significant difference between the concentrations of the same extract (p<0.05, ANOVA, DMRT); EtAI = Ethanol extract of *A. indicum*.

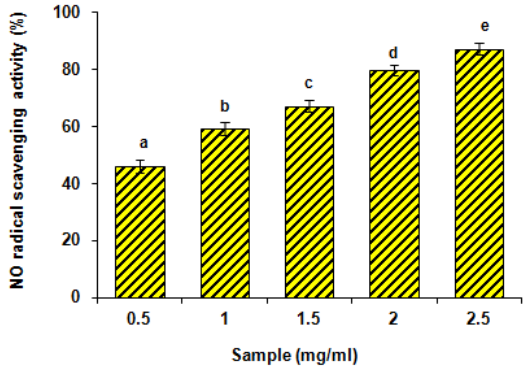

Figure 6: Nitric oxide radical scavenging assay of EtAI. Each value is expressed as mean±SD (n=3). Different letters (a-e) indicate a significant difference between the concentrations of the same extract (p<0.05, ANOVA, DMRT); EtAI = Ethanol extract of *A. indicum*.

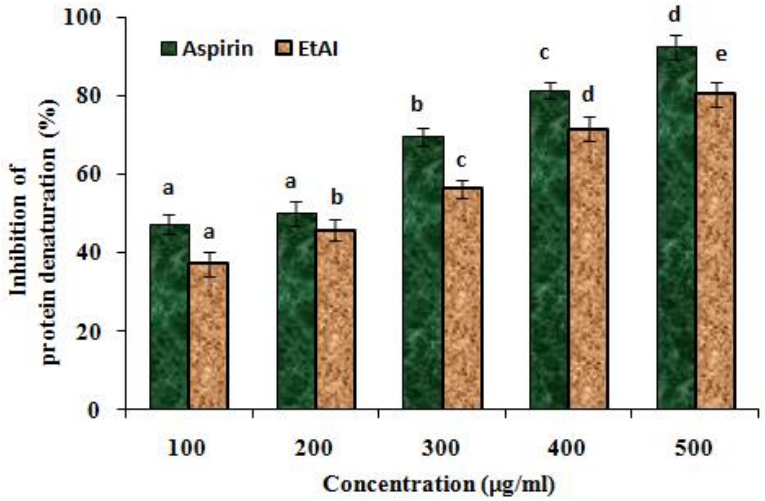

Figure 7: *In vitro* anti-inflammatory effect of EtAI. Each value is expressed as mean±SD (n=3). Different letters (a-e) indicate a significant difference between the concentrations of the same extract (p<0.05, ANOVA, DMRT); EtAI = Ethanol extract of *A. indicum*.

Statistical analysis

All analysis was carried out in triplicates and results are given as mean±SD. Analysis of variance and Duncan's multiple range test were used to find out the differences among scavenging and anti-inflammatory activities at different concentration EtAI for different assays with least significance difference, $p<0.05$ as a level of significance. The analysis was done using SPSS software.

RESULTS AND DISCUSSION

Preliminary phytochemical screening

Plants are enriched with free radical scavenging molecules such as phenolic acids, lignins, vitamins, terpenoids, tannins, coumarins, alkaloids, flavonoids, quinines and other secondary metabolites. Studies proved that, these antioxidant compounds have antiatherosclerotic, antitumor, anti-inflammatory, antibacterial, antiviral and antimutagenic activities[21]. The preliminary phytochemical screening studies may be useful to identify the bioactive principles present in the plants and subsequently may lead to the development of drug discovery[22]. The extraction of phytochemical compounds from the plant depends on the diffusion of each compound into the solvent. The factor that needs to be considered when selecting the solvent for phytochemicals extraction is the solubility of the target compound[23, 24].

In the present study, phytochemical screening was conducted on AqAI, EtAI, AcAI, PeAI and EaAI extracts to find out the existence of certain bioactive compounds such as flavonoids, triterpenoids, phenols, carbohydrates, alkaloids, proteins, glycosides, steroids and saponins and the results were depicted in table 1. AqAI revealed the presence of phenols, flavonoids, proteins, triterpenoids and steroids. EtAI showed the presence of Alkaloids, phenols, flavonoids, proteins, carbohydrates, triterpenoids and saponins. AcAI showed the presence of phenols, flavonoids and saponins. Alkaloids, phenols, triterpenoids and saponins were present in PeAI. EaAI revealed the presence of alkaloids, phenols, flavonoids, carbohydrates and saponins. EtAI was found to possess more bioactive compounds compared to other extracts. Thus further studies were carried out on EtAI.

Fourier-transform infrared spectroscopy (FT-IR)

FT-IR spectrum analysis was conducted in order to investigate the molecular properties and functional groups of EtAI. The presence of different functional groups made the EtAI spectra very complex (Fig. 1). The absorption peaks corresponded to 3873-3000 cm^{-1} was for O-H stretching, 3000-2800 cm^{-} was for C-H stretching vibrations, 1750-1650 cm^{-1} was for the stretching of C=O bonds in acids and esters[25], the amide I and amide II absorptions of proteins (1650-1450 cm^{-1}), esters and aliphatic chains of fatty acids (1460-1150 cm^{-1}) and C=O and C-C stretching of acids (1200-800 cm^{-1}), The strong absorption peak at 2330 cm^{-1} was due to the stretching vibration of O=C=O. The broad absorption peak at 1921 cm^{-1} was due to the transition metal carbonyls (C≡C stretching). The very intense peak at 1049 cm^{-1} indicated C-N stretching (primary amines). The absorption peak near 879 cm^{-1} was for hydrogen-bonded O-H out-of-plane bending[26].

Determination of total phenol and flavonoid content

The flavonoid and phenolic compounds can be used as important indicators of free radical scavenging capacity which can be used as a preliminary screening for any natural source of antioxidants[27]. Flavonoids and phenolics are a major group of compounds that act as free radical scavengers or primary antioxidants[28]. Total phenolic and flavonoid content of EtAI was found to be 5.82 mg CE/g, 7.91 mg RE/g respectively (Fig. 2). These phytochemicals are responsible for their medicinal value.

Total antioxidant capacity (TAC) assay

Total antioxidant capacity of EtAI was studied using phosphomolybdenum method and this method is based on the principle that the antioxidants present in the extract reduce molybdenum and form green molybdenum (V) complex. The TAC of EtAI was found to be 28.57 mg AAE/g (Fig. 2). Thus, the bioactive principles or phytochemical compounds such as phenols and flavonoids are responsible for the total antioxidant activity of EtAI.

ABTS radical scavenging assay

The $ABTS^{\bullet+}$ radical scavenging assays are widely used methods for the assessment of the antioxidant capacities of any natural products. It is a spectrometric technique based on quenching of stable colored radicals ($ABTS^{\bullet+}$) using potassium persulphate[29]. The extent of decolorization is determined as percentage inhibition of the ABTS radical cation as a function of concentration. EtAI strongly exhibited ABTS radical scavenging activity in a concentration-dependent manner. The percentage of ABTS radical scavenging activity by EtAI is shown in Fig. 3. The scavenging activity was found to be statistically significant ($p<0.05$) and it ranged from 34.46% to 71.38% at 1 to 5 mg/ml. The EC_{50} value was 2.65 mg/ml. This study suggests that EtAI possess excellent scavenging effect against ABTS radical.

Hydroxyl radical scavenging activity

The hydroxyl radical is considered to be the most poisonous free radical because it can oxidize biological macromolecules and cause lipid peroxidation which in turn affects cell membrane's permeability and integrity[30]. Most of the hydroxyl radicals are produced from the decomposition of hydro peroxides (ROOH). EtAI exhibited antioxidant activity over a concentration range of 1.5 to 7.5 mg/ml with an scavenging effect of 40.64% to 75.49% (Fig. 4) and the difference was statistically significant ($p<0.05$). The EC_{50} value was found to be 3.66 mg/ml. The antioxidative components in EtAI are responsible to scavenge hydroxyl radicals.

Reducing power

Antioxidants donate electrons to reactive free radicals and reduce them into more stable and unreactive agents[31]. Reducing agents or antioxidants can inhibit lipid peroxidation reactions which causes cellular membrane damage[32]. The reduction capacity of a compound in the extract may serve as a significant indicator of its potential antioxidant activity. The reducing power of EtAI was 0.261 at 0.8 mg/ml and 1.068 at 4 mg/ml (Fig. 5). There was a significant difference in reducing power between the concentrations tested ($p<0.05$) and the EC_{50} value was 1.94 mg/ml. Hence, EtAI can be considered as reducing agents, which could react with free radicals to hinder lipid peroxidation reactions.

NO radical scavenging assay

NO is synthesized in biological tissues and is responsible for various inflammatory reactions in cells[33]. Nitric oxide reacts with oxygen and produces the stable products, nitrates and nitrites. The excess concentration of these stable products is responsible for the progression of several diseases[34]. In the presence of free radical scavenger, the production of nitrates or nitrites will decrease[35]. Fig. 6 represented the NO scavenging activity of EtAI. It is to be noted that EtAI exhibited a greater NO scavenging activity of 87.25% at 2.5 mg/ml. There was a significant difference in scavenging activity between the concentrations tested ($p<0.05$) and the EC_{50} value was 0.62 mg/ml. The antioxidant principles present in EtAI might be responsible for the observed nitric oxide scavenging activity.

***In vitro* anti-inflammatory activity (Protein denaturation method)**

Denaturation of tissue protein is a well-known cause of inflammation. The denatured protein expresses antigens (auto antigens) which are associated to type III hyper-sensitive reaction and causes life threatening diseases[36, 37]. In the present investigation, the *in vitro* anti-inflammatory activity of EtAI was evaluated against denaturation of protein (Bovine serum albumin). At the concentration range of 100 - 500 µg/ml, EtAI and aspirin exhibited concentration dependent inhibition of protein (albumin) denaturation (Fig. 7). There was a significant difference in inhibition of protein denaturation between the concentrations tested ($p<0.05$). The IC_{50} value was 227 µg /ml for EtAI and 209 µg /ml for aspirin. Thus, EtAI could control the production of auto antigen and can act as an effective anti-inflammatory agent. The secondary metabolites like flavonoids, phenolics present in EtAI might be responsible for this activity.

CONCLUSION

In conclusion, the antioxidant and anti-inflammatory activity of ethanolic extract of *A. indicum* was evaluated using various *in vitro* biochemical tests. It possessed strong free radical scavenging and anti-inflammatory properties with considerable flavonoid and phenolic content. Thus, the present investigation clearly suggesting that *A. indicum* have the ability to control cellular oxidative stresses and can acts as a natural antioxidant.

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