IJBPAS, August, 2017, 6(8): 1562-1577

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

EFFECT OF EXTRACTION SOLVENT ON TOTAL PHENOL CONTENT, TOTAL FLAVONOID CONTENT, ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITY OF *ARTABORTRYS HEXAPETALUS*

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Received 7th Nov. 2016; Revised 5th Jan. 2017; Accepted 2nd April 2017; Available online 1st August 2017

ABSTRACT

Artabortrys hexapetalus (L.f.) Bhandari belongs to family annonaceae .*A. hexapetalus* is flowering biennial plant native to southern part of china and asia and commonly used for the treatment of malaria and scrofula. **Objective:** The present study designed to perform phytochemical screening and to evaluate the total phenolic content, total flavanoidal content, *invitro* antioxidant and anti-inflammatory activities of different extracts of aerial parts of Artabortys hexapetalus. **Method:** All the successive extracts (petroleum ether, chloroform, ethanol and aqueous extracts) were obtained by hot extraction method and were subjected to preliminary phytochemical screening. Total phenolic content was estimated by folin-ciocalteu reagent, flavanoid content by aluminium chloride reagent and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) were used to determine in-vitro antioxidant activity of plant extracts. Anti- inflammatory activity was investigated by albumin denaturation and protein inhibitory activity. **Result:** The highest extraction yield was obtained by using ethanol. Phytochemical analysis of all the extracts revealed the presence of major classes of phytochemicals such as alkaloids tannins, steroids etc. Good antioxidant activity was found with considerable amount of total phenolic and flavonoid content. The anti-inflammatory activity of ethanolic extract was significant and comparable with

the standard anti-inflammatory drug, diclofenac. **Conclusion:** Based on the result of this pilot study, it can be concluded that *A. hexapetalus* is a good source of natural antioxidants which can be used to prevent progression of many chronic diseases. Further detailed phytochemical studies are needed to identify the chemical compounds responsible for exhibiting potent anti-inflammatory activity.

Keywords: Solvent Extraction, Total Phenol Content, Total Flavonoid Content, Antioxidant activity, Anti-Inflammatory Activity, *Artabortrys Hexapetalus*

1. INTRODUCTION

Oxygen is an element indispensable for life and necessary for the generation of energy. Free radicals are released as a consequence of incomplete reduction of oxygen during ATP production in the mitochondria. The free radical include reactive oxygen species (ROS), reactive nitrogen species (RNS), and free radicals are generated in living organisms by a variety of endogenous systems (e.g., respiration, oxidative energy metabolism, immune activity), and after exposure to different physical and chemical factors (e.g., UV radiation, pesticides, pollutants, drugs, food additives) [1]. At lower concentration, ROS and RNS exert beneficial effects on cellular responses and immune functions but overproduction of such free radicals can generate oxidative stress . Oxidative stress causes oxidative damage to biomolecules (e.g. lipids, proteins, DNA), eventually leading to many chronic diseases, such as atherosclerosis, cancer, diabetes, aging, and other degenerative diseases in humans [2]. Human body has several mechanism to counter act oxidative stress by producing enzymes such as superoxide dismutase, catalase and by antioxidant compounds such as ascorbic acid, tocopherols and gluthatione, which are either naturally produced in situ or externally supplied through foods [3]. In addition, supplementation with exogenous antioxidants or boosting of endogenous antioxidant defenses of the body has been found to be a promising method of countering the undesirable effects of oxidative stress [4].

Many human diseases like diabetes mellitus, cancer, atherosclerosis, arthritis, and neurodegenerative diseases, inflammatory diseases have been correlated with oxidative damage to various biomolecules including, proteins, lipids, lipoproteins and DNA. When the mechanism of antioxidant protection becomes unbalanced, polymorphonuclear leukocytes, macrophages and peroxisomes are stimulated, the result may be the above-

mentioned diseases and accelerated aging [5, 6]. The immune system is often involved in inflammatory disorders and is demonstrated in both allergic reactions and myopathies. Non-immune diseases with inflammation include atherosclerosis and ischemic conditions [7]. Inflammatory responses involve release of chemo-attractant mediators and chemo-activators, enzyme activation, tissue break down and repair. But inflammation may also be responsible for serious fatal situation [8]. A number of synthetic drugs particularly non-steroidal antiinfllammatory drugs (NSAIDS) available act by inhibiting the function of prostaglandin synthesis in the inflamed tissue. The other class of drug is glucocorticoids e.g. cortisone and prednisone etc act by binding to cortisol receptors and reduce the inflammation [9]. All of these drugs have undesirable side effects on the contrary many medicines of plant origin had been used since long time with little side effects as they contain bioactive compounds such as phenols, alkaloids, tannins, steroids and so forth, most commonly.

Artabotrys hexapetalus [(L.F) bhandari] (Annonaceae) a medicinal plant as a whole was commonly known as 'hari champa' is widely distributed throughout the southern part of china and also in the southern part of asia [10]. In china, its roots and fruits are used for treating malaria [11], scrofula[12] and anti-fertility activity [13]. The flower is acrid, bitter, alexiteric, useful in vomiting, biliousness, diseases of the blood and the heart, itching, sweating, bad breath, thirst, leucoderma, headache, disease of the bladder [14]. A wide range of chemical compounds including alkaloids, sesquiterpenes, flavanoids, fixed oils and volatile oils have been found to posses various pharmacological activities [15].

The basic aim of the research was to determine the total phenolic and flavonoidal content in various extracts of the species *A. hexapetalus* using spectrophotometric methods, as well as to examine antioxidant and anti-inflammatory activity of plant extracts using different *in vitro* models.

2. MATERIALS AND METHODS

2.1. Drugs and chemicals

DPPH, ABTS, gallic acid, quercetin were purchased from Sigma-Aldrich USA. Folin-Ciocalteu reagent, Aluminium chloride and Ascorbic acid were obtained from Merck, Germany. All other chemicals used in the study were of analytical grade.

2.2. Collection of plant material

Plant materials (leaves, twigs, aerial parts and roots) were collected from the nursery of Roshanara Bagh, New Delhi between

November 2013 and January 2014. Voucher specimen was authenticated by Dr. Sunita Garg, Scientist F and Head, Raw Material Herbarium and Museum, NISCAIR (National Institute of Science Communication and Information Resources) Pusa Gate, New Delhi. The voucher specimen (NISCAIR/RHMD/consult/2014/2546/125) of the test drug was deposited in the NISCAIR herbarium for future reference. The aerial parts were cleaned and washed under tap water and dried under shade. The dried samples were powdered in a grinder and kept in air tight.

2.3. Sample preparation

The fresh aerial parts were dried under the shade and powdered in a mixer. The coarse powdered material (500g) of *A. hexapetalus* was extracted successively by petroleum ether, dichloromethane, ethanol and distilled water in SOXHLET apparatus. Each time before extraction with next solvents, the coarse powder material was dried in hot air oven below 50ºC. The extracts were evaporated to dryness under reduced pressure with a rotary evaporator (Heidolph) at a temperature of 40^{o}C while the water filtrate was freeze-dried to powder. Yields for each extraction are indicated in Table 1. All the dried extracts were kept in tightly packed container under refrigeration until used for the biological testing.

2.4 Phytochemical screening of extracts

The freshly prepared crude extracts of aerial parts of *S. alata* were subjected to qualitative phytochemical tests to check the presence of various classes of active chemical constituents such as tannins, saponins, glycosides, flavonoids, alkaloids, terpenes, steroids, etc. using standard procedure. (16)

2.5 Determination of total phenolic contents in the plant extracts

The concentration of phenolics in plant extracts was determined with the folin ciocalteau reagent using the method of spanos and wrolstad (1990) as modified by Lister and Wilson (2001) (17, 18). All the extract in the concentration of 1 mg/mL was used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of solution of extracts, 2.5 mL of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 mL 7.5% NaHCO3. Blank was concomitantly prepared**,** containing 0.5 ml methanol, 2.5 mL 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 mL of 7.5% of NaHCO3. The samples were thereafter incubated in a thermostat at 45^{o}C for 45 min. The absorbance was determined using spectrophotometer at λmax = 765 nm. The samples were prepared in triplicate for each

analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid and the calibration line was construed. Based on the measured absorbance, the concentration of phenolics was read (mg/mL) from the calibration line; then the results were expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g dw).

2.6 Determination of flavonoid concentrations in the plant extracts

The content of flavonoids in the examined plant extracts was determined using spectrophotometric method (19). The sample contained 1 mL of methanolic solution of the extracts in the concentration of 1 mg/mL and 1 mL of 2% AlCl3 solution dissolved in methanol. The samples were incubated for an hour at room temperature. The absorbance was determined using spectrophotometer at λmax = 415 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of quercetin and the calibration line was construed. Based on the measured absorbance, the concentration of flavonoids was read (mg/mL) on the calibration line; then, the content of flavonoids in extracts was expressed in terms of quercetin equivalent (mg of QU/g of extract).

2.7 In-vitro Anti-oxidant activity

2.7.1 DPPH radical scavenging activity

The ability of the plant extract to scavenge 1, 1-diphenyl-2-picryhydrazyl (DPPH) free radicals was assessed by the standard method (20). The stock solution of extracts were prepared in ethanol to achieve the concentration of 1 mg/ml. Dilutions were made to obtain concentrations of 20, 40, 60,80 and 100 µg/mL. Diluted solutions (1 mL each) were mixed with 3 ml of ethanolic solution of DPPH (DPPH, 0.004%). After 30 min of incubation at room temperature the reduction of the DPPH free radical was measured by reading the absorbance at 517nm using UV-Visible Spectrophotometer. Initially, absorption of blank sample containing the same amount of ethanol and DPPH solution was prepared and measured as control. Ascorbic acid was used as standard. The experiment was carried out in triplicate. Percentage inhibition was calculated using equation (1), whilst IC50 values were estimated from the % inhibition versus concentration plot, using a non-linear regression algorithm. The data were presented as mean values ± standard deviation (n = 3).

$$\% \text{ inhibition} = \frac{(\text{Absorbance of control} - \text{absorbance of sample})}{\text{Absorbance of control}} \times 100 \quad \text{equation (1)}$$

2.7.2 ABTS radical scavenging assay

For ABTS cation radical scavenging assay, the procedure followed the method with some slight modifications (21, 22). The stock solutions included 7mM $ABTS^+$ solution and 2.4mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12h at room temperature in the dark. The solution was then diluted by mixing 1ml $ABTS^+$. Solution with 60 ml methanol to obtain an absorbance of 0.706±0.001 units at734nm using the spectrophotometer. Fresh $ABTS^+$ solution was then prepared for each assay. Plant extracts/standard (1ml) of different concentration (20, 40, 60, 80,100 and 200 µg /mL) was allowed to react with 1ml of the $ABTS^+$ solution and the absorbance was taken at 734nm after 7min using the spectrophotometer. The $ABTS^+$ scavenging capacity of the extract was compared with that of Trolox and percentage inhibition calculated as:

ABTS radical scavenging activity (%) = $Abs_{control} - Abs_{sample}/Abs_{control} \times 100$ Where,

$Abs_{control}$ was the absorbance of ABTS radical + methanol, and

Abs_{sample} was the absorbance of ABTS radical + sample extract/standard.

2.8 Anti-inflammatory activity

2.8.1 Inhibition of albumin denaturation

The anti-inflammatory activity of all the extracts were studied by using inhibition of albumin denaturation technique which was studied according to Mizushima et al (23) and Sakat et al (24) followed with minor modifications. The reaction mixture was consists of test extracts and 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was adjusted using small amount of 1N HCl. The sample extracts were incubated at 37 ºC for 20 min and then heated to 51 º C for 20 min, after cooling the samples the turbidity was measured at 660nm. The experiment was performed in triplicate. The Percentage inhibition of protein denaturation was calculated by equation 1.

2.8.2 Protein Inhibitory Activity

This test was done according to the method described (25) with slight modifications. The reaction mixtures contained 0.5 mL trypsin and chymotrypsin (8.000 Armour units of enzyme activity), 1.0 mL 25 mM trisHCI buffer (pH 7.4) and 1.0 mL extracts of *S.alata* (20-100 µg/mL) and standard diclofenac sodium (20-100 µg/mL). The mixtures were incubated at 37 °C for 5

minutes. Then 1.0 mL of 0.8% (w/v) casein was added. The mixtures were incubated for the additional 20 minutes. 2.0 mL of 70% perchloric acid was added to terminate the reaction. Cloudy suspension was centrifuged. Absorbance of the supernatant was measured at 280 nm against buffer as a blank. Protein inhibitory activity (in %) is calculated as follows:

Protein inhibitory activity (%) = [100 (O.D. of control O.D. of test) / O.D. of control] x 100

where O.D. is optical density

Data analysis

All experiments measurements were carried out in triplicate and are expressed as mean ± SD. The inhibitory concentration 50% (IC50) was calculated by plotting the data in the graph as concentration versus percentage inhibition using graph pad prism software, version 5.

3. RESULTS

3.1 Result of Preliminary analysis

Preliminary phytochemical investigation revealed the presence of plants secondary metabolites such as carbohydrates, alkaloids, saponins, tannins, glycosides, flavonoids and steroids. The results are summarised in Table 1 where + and - signs represents the presence and absence of the compounds repectively.

3.2 Total phenolic content (TPC) and flavanoidal content (TFC)

The TPC and TFC of all the extracts were expressed in terms of gallic acid, quercetin and yield (%) w/w are presented in Table 2. The TPC and TFC were calculated using the following linear regression equations based on the calibration curves of gallic acid (1) and quercetin (2).

$y=0.005x-0.007$, $r^2 = 0.991$ (1)

$y=0.001x+0.092$, $r^2=0.958$ (2)

ETAH extract found to contain higher amount of phenolic and flavanoidal compounds as compared to other extracts. However, direct relationship in case of ETAH extract and inverse relationship in case of AQAH was observed in yield (%).

3.3 Result of DPPH free radical scavenging activity of *A. hexapetalus*

The relatively stable DPPH radical is widely used to evaluate the free radical scavenging activity of various natural antioxidants including plant extracts. The data presented in Figure 1 showed the percent inhibition of DPPH radical scavenging activity of different extracts of *A. hexapetalus*. The ethanolic extract (ETAH) exhibited higher antioxidant activity with IC50 value of 31.03±2.19 µg/ml as compared to ascorbic acid (24.46±0.67 µg/ml), IC50 value of PEAH (237.39±4.66 µg/ml), DCMAH (193.31±0.72 µg/ml) and AQAH (185.42± 2.41 µg/ml) extract failed to show any significant scavenging activity.

From the data, we observed that DPPH radical scavenging activity was increased as the concentration increased for each individual extract, with marked increase in methanol extract (Figure 1).

3.4 Result of ABTS reducing activity of *A. hexapetalus*

The ABTS scavenging activity of all the extracts of *A. hexapetalus* were detected and compared with Trolox. The results are expressed as percentage inhibition (% inhibition) at various concentration (20-200 µg/mL) of PEAH, DCMAH, ETAH, AQAH and Trolox (standard). The IC50 values are calculated from graph and were found 73.02±0.73, 126.19±0.88, 163.78±1.85, 81.17±1.05,158.93±1.06µg/ml for Trolox, PEAH, DCMAH, ETAH and AQAH respectively. The IC50 of DCMAH, PEAH and AQAH are significantly lower than IC50 of Trolox ($p<0.05$) is considered significant while the IC50 of ETAH ($p<0.001$) is comparable with IC50 of Trolox. Results are given in figure 2.

3.5 Result for Protein denaturation

The data in Figure 3 showed that all the extracts PEAH, DCMAH, ETAH, AQAH and (Asprin) inhibited significantly thermally induced protein denaturation in a concentration dependent manner. It also showed that ETAH extract had the highest (IC50=71.37±0.30µg/mL) denaturation of protein activity while aqueous fractions had the lowest (IC50=106.55±1.23 µg/mL) anti-denaturation of protein activity (Table 3).

3.6 Result for Antiproteinase activity

The *A. hexapetalus* crude extract exhibited significant antiproteinase activity from different extracts. As shown in figure 4 the maximum inhibition was observed from ETAH and DCMAH extract and minimum with PEAH and AQAH. The standard Diclofenac drug showed the maximum proteinase inhibitory action as per Table 3. The IC50 of PEAH, DCMAH, AQAH are significantly lower than IC50 of Diclofenac ($P<0.05$) and are considered significant while the IC50 of ETAH is comparable to IC50 of diclofenac and considered non significant. All the extract showed significant inhibition in dose dependant manner.

Table 1: Phytochemical screening of extracts

TEST	Petroleum ether Extract	DCM Extract	Ethanolic Extract	Aqueous Extract
Alkaloids	-	+	+	+
Flavanoids	-	+	+	+
Tannins	-	-	+	+
Saponins	-	-	+	+
Glycosides	-	-	+	+
Steroids	-	+	+	+
Steroidal terpenes	+	-	-	-
Phenolic	-	-	+	+
Gums and mucilage	-	-	-	+
Carbohydrates	-	-	+	+

+ =Present, - =Absent

Table 2: Result for Total Phenolic and flavanoidal content of extracts

Extract	Yield (%) (w/w)	mg of GA/g of extract	mg of Quercetin/g of extract
PEAH	1.499	24.99 ± 1.60	2.43±0.50
DCAH	0.892%,	41±1.22	21.73±29.9
ETAH	7.2%	142.76±0.89	48.03±1.45
AQAH	10.18%.	63.33±1.8	50.55±0.964

Table 3: Fifty percent inhibitory concentration (IC50) of *A. hexapetalus* on protein denaturation and anti-proteinase activity

Extracts of *A. hexapetalus*	IC50 (µg/mL)	
	Protein denaturation	`Antiproteinase activity
ASPRIN/Diclofenac	68.05±1.75	60.71±0.14
PEAH	101.91±1.41***	130.11±2.54***
DCMAH	89.01±0.62***	66.41±0.56**
ETAH	71.37±0.30*	63.49±0.74^{ns}
AQAH	106.55±1.23***	143.99±0.45***

Each value represents the mean ± SD, n=3, and results were analyzed by one way ANOVA followed by Dunnet test. ***p<0.0001, considered extremely significant, **p<0.001, p<0.01, p<0.05 are considered significant; ns p>0.05, non significant

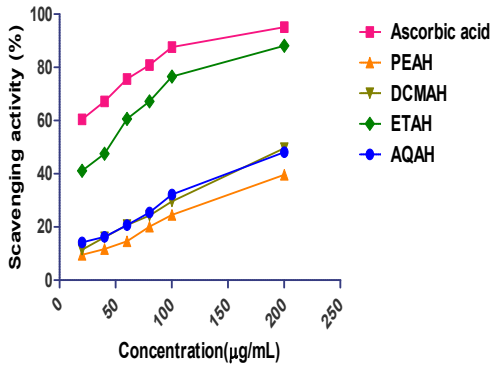

Figure 1: Showing DPPH scavenging activity of extracts

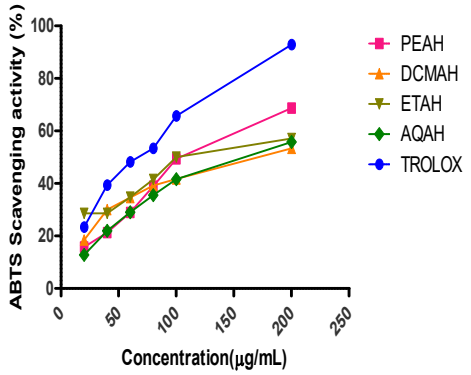

Figure 2: Showing ABTS Scavenging activity of extracts

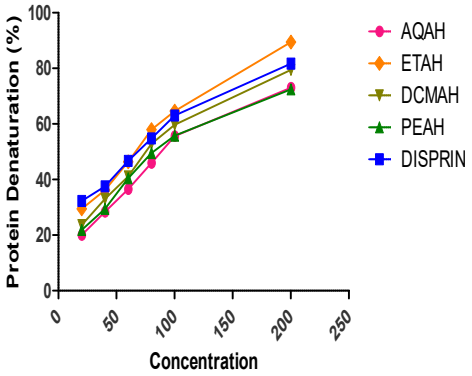

Figure 3: Showing Protein denaturation of extracts

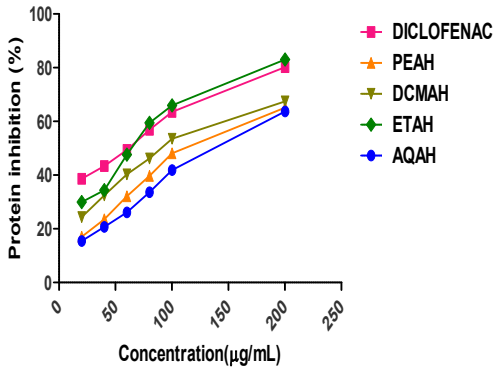

Figure 4: Showing Protein inhibition of extracts

DISCUSSIONS

The inherent problems associated with the use of animals at the initial stage of drug discovery such as lack of rationale for their use when other suitable methods are available, ethical issues and difficulty with cross species extrapolation led us to adopt *in vitro* methods such as DPPH, ABTS, anti-denaturation of protein and anti-proteinase bioassays for the assessment of antioxidant, anti-inflammatory property of *A. hexapetalus*. In the current study, a sequential extraction involving the solvent of increasing polarity to extract the bioactive compounds was used. It is found that percentage yield in ETAH extract was more because the nature, polarity, and the solubility of the bioactive compounds in *A. hexapetalus.*

The various extracts of *A. hexapetalus* were tested for different phytoconsituents like alkaloids, glycosides, saponinins, tannins, terpinoids, reducing sugars, phenolic compounds, flavanoids, protein, carbohydrates and volatile oils. The Knowledge of the phytoconstituents in plants is desirable because such information will be valuable for synthesis of complex chemical substances and to screen for biological activities [26].

Flavonoid and phenolic compounds are widely distributed secondary metabolites in plants having potent antioxidants which prevent oxidative cell damage and also possess anti-inflammatory, anti-allergic and anti-thrombotic[27, 28]. Our data initially indicated that ETAH extract of *A. hexapetalus* not only showed significant presence of polyphenols but also exhibited the highest amount of TPC and TFC than other extracts, which is in well agreement with earlier studies [29, 30]. In the quantitative assay the most promising antioxidant potential was observed with the

ETAH extract. This indicates that the plant extracts are electron donors so they can react with free radicals, convert them into more stable products and terminate the radical chain reaction. This may be important in protecting cellular DNA, lipids and proteins from free radical damage [31]. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity and generally correlates with the presence of reductones, which have been shown to exert antioxidant activity by breaking the free radical chain and donating a hydrogen atom [32]. Our results demonstrated that *A. hexapetalus* ethanolic extract exhibited strong DPPH radical and ABTS scavenging activity with respect to other extracts (Figure 1 and 2), which in turn signifies its potent antioxidant activity. It was observed that some phytochemicals such as alkaloids, flavonoids, glycosides, steroids and saponins were commonly present in all genus of annonaceae and some were found species specific [33-35]. So it is assumed that the antioxidant potential may be due to the common presence of various secondary metabolites.

Phenolic compounds have been said to account for most of the antioxidant activities of plant extracts [32], it is well known that compounds capable of scavenging free radicals can delay, inhibit, or prevent the oxidation of various biomacromolecules and diminish the oxidative stress, which play major role in the development of several inflammatory diseases like arthritis [36]. Denaturation of proteins is responsible for the cause of inflammation in conditions like rheumatoid arthritis hence by, prevention of protein denaturation may also help in preventing inflammatory conditions. NSAIDS acts in similar way in preventing inflammation [37]. Proteinases are involved in arthritic reactions. The main source of proteinase is the lysosomal granules of neutrophils. Earlier it was reported that leukocytes proteinase play important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors [38]. The in vitro anti-inflammatory effect of *A. hexapetalus* extracts was evaluated against albumin denaturation assay ETAH and DCMAH showed highest activity among all other extracts and as compared to Asprin as given in Table 3. In antiproteinase assay ETAH and DCMAH showed promising activity as compared to the standard Diclofenac. It was evident by IC50 values as given in Table 3.

Alkaloids are known anti-inflammatory effects [39]. Flavonoids and related

polyphenolic contributes significantly to the antioxidants properties which prevent oxidative cell damage and also possess anti-inflammatory, anti-allergic and anti-thrombotic [40, 41]. Hence, the presence of alkaloids and polyphenolic compounds in ETAH may be contributing for its anti inflammatory activity. The anti-inflammatory activity is may be due to inhibition of enzymes responsible for the production of chemical mediators of inflammation and arachidonic acid metabolism [42].

CONCLUSION

Exploration of the in vitro pharmacological activity of from Artabotrys hexapetalus extracts revealed that alkaloids and phenolic compounds may have potential therapeutic agent in view of its dual-acting antioxidant and antiinflammatory properties. Hence, further studies are required to elucidate the mechanisms underlying the observed inhibitory effect of various extracts.

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