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Petroleum Ether and Chloroform Soluble Fractions of Whole Plant Extract of *Acanthus ilicifolis* Linn. Possesses Potential Analgesic and Antioxidant Activities

Md. Abu Rayhan Faysal, Md. Abdul Barek, Sharmin Jahan, A. F. M. Shahid-Ud-Daula, Mohammad Anwarul Basher, Md. Hasanuzzaman*

ABSTRACT

Background: Medicinal plants are the major sources of traditional treatment of disease and new drug discovery due to major side effects of synthetic drug.

Objectives: The aim of study was to evaluate analgesic and antioxidant activities of petroleum ether and chloroform soluble fractions of whole plant extract of *Acanthus ilicifoius*.

Materials and Methods: The plant extract, standard diclofenac, and distilled water as control was administered post orally in Swiss albino mice and observe the analgesic activity by acetic acid (0.6%) induced writhing method. The plant extract was also subjected to perform reducing power assay, DPPH free radical scavenging activity, and FRAP assay to evaluate antioxidant activity.

Results: The pet ether, and chloroform soluble fraction of plant extract revealed significant analgesic activity on mice model. Notably, the pet ether and chloroform fraction showed (40.14 ± 2.32) % and (40.12 ± 0.9) % analgesic inhibition, whereas standard diclofenac revealed (52.79 ± 2.62) % analgesic inhibition. In antioxidant activity assay, the plant extract showed mild to moderate antioxidant activities compare to standard ascorbic acid.

Conclusion: From the results, it could be concluded that, the pet ether and chloroform fractions of whole plant extract of *A. ilicifolius* possesses potential analgesic and antioxidant properties.

Key Words: *Acanthus ilicifolius*, analgesic activity, antioxidant activity, petroleum ether, chloroform, DPPH.

*Correspondence to:
Md. Hasanuzzaman, M.Pharm, PhD, Associate Professor, Department of Pharmacy, Faculty of Science, Noakhali Science and Technology University, Noakhali-3814, Bangladesh.
hasanprdu@gmail.com

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INTRODUCTION

The ancient people use medicinal plants in the treatment of various diseases without proper therapeutic knowledge. But, now a days, many research is going on in the basis of natural medicinal plants to determine the pharmacological actions such as antioxidant, antimicrobial, analgesic, antipyretic, sedative, antidepressant, anxiolytic, antipsychotic, anticancer, anti-diabetic and other different activities.[1] *Acanthus ilicifolius* Linn. (Acanthaceae) is a perennial vascular plant, popularly recognized as "Holy leaved acanthus». Historically, the plant is employed in traditional systems of medicine, including traditional Indian medicine or ayurveda and in traditional Chinese medicine for the treatment of various ailments.[2,3] The different parts of plant of *A. ilicifolius* such as fruits, leaves, bark, and roots are reported to possess various bioactive compounds such as triterpenoids, alkaloids, phenolic compounds, lignin, flavonoid, steroids, and terpenoids. Thus, the plant part or whole plants are reported to use in asthma, diabetes, hepatitis, inflammatory disease, and rheumatoid arthritis.[2,4,5] Methanolic extract of leaves of *A. ilicifolius* has been reported to exert hepatoprotective and tumor suppressive activities.[6,7] The plant parts have been used for the treatment of asthma, diabetes, dyspepsia, leprosy, hepatitis, paralysis, rheumatoid arthritis and dieresis.[4,8] Besides, the plant has been shown to possesses anti-inflammatory, antioxidant, antileishmanial, osteoblastic, hepatoprotective, anticancer, antiulcer and antimicrobial activities.[9] The plant is wealthy in bioactive compounds and due to possess of biological activities the plant is frequently being investigated. According to Worlds Health Organization (WHO), more than 80% of world inhabitants depends on using plant for their medicine and mangroves have been widely used for that purpose.[4] *A. ilicifolius* is a true mangrove species of plant and found in Asian tropics from India to Bangladesh, mangrove forest of Srilanka, Polynesia and northern Australia.[10,11]

Conventional non-steroidal anti-inflammatory drugs are common in the treatment of pain, bruise and inflammation. But, several adverse reactions are associated with these agents limiting the widespread uses of the agents. Development of newer and safest anti-inflammatory compound possessing

Department of Pharmacy, Faculty of Science, Noakhali Science and Technology University, Noakhali-3814, Bangladesh

fewer side effects is a challenge to the scientific community. However, to search new anti-inflammatory components from medicinal plant is ongoing and having a great interest of scientists.[5]

Free radical and reactive oxygen species are responsible for oxidative stress and different pathogenesis such as inflammation, arthritis, diabetes, parkinson's, alzheimer's disease, as well as cancer and atherosclerosis. Those components are also responsible for human aging.[12,13] The antioxidant molecules are defined as any substances that delays or inhibit oxidative damage to a target molecules.[14] They can trap free radical, peroxide, hydroperoxide or lipid peroxyl molecules to prevent oxidative damage of cells and protect from degenerative diseases.[15] The use of synthetic antioxidant is an old practice, however several question may arise in consumer concerning safety issues. Thus, alternative or natural source of antioxidant from medicinal plants are attracting consumers.[16]

Despite its wide anecdotal uses, few pharmacological studies on whole plant have been performed. The aim of our research was therefore to examine the effect of petroleum ether (pet ether) and chloroform soluble fractions of whole plant extract of *A. ilicifolius* on anti-oxidant and analgesic activity.

MATERIALS AND METHODS

Plant Materials and Extract Preparation

The whole plant of *A. ilicifolius* was collected from Sundarban, a mangrove forest situated in the southwest coastal region of Bangladesh. The plant specimen was subjected to National Herbarium, Dhaka for identification. The experience taxonomist identified the plant sample and provided a identification number (accession number: Acc-46522). The plant was thoroughly washed, sun dried and powdered by rotary cutter mill. About 800 g of dried powder was taken in two clean, flat bottomed glass containers and soaked in 1500 ml of pet ether and 1500 ml of chloroform, respectively. The soaked plant extract was kept at room temperature for two weeks with occasional shaking and stirring. The whole mixture was filtered using filter cloth following a Whatman filter paper (Bibby RE200, Sterilin Ltd., UK). The filtrate was evaporated by using rotary evaporator (RE-EV311-V, LabTeck S.R.L, Italy) and then kept under ceiling fan for several days. It rendered a gummy concentrate of greenish black color. The gummy concentrate was designated as pet ether and chloroform soluble fraction and the extract was kept at 4 °C for further analysis.

Experimental Animals

The Swiss albino mice of both (male and female) sex weighing 20–30 g and aged 6–8 weeks were purchased from the animal house of the Department of Pharmacy, Jahangirnagar University, Dhaka-Bangladesh. All of the animals were kept in plastic cages at room temperature and on a 12 h light-dark cycle. The animal had free access to standard pet diet (pellet food) and water *ad libitum*. The experiment was done in the Physiology Laboratory of the Department of Pharmacy at Noakhali Science and Technology University. The mice were acclimatized to laboratory environment for 1 week prior to the experiment. Standard pet diet was withdrawn 18 h prior to the beginning of experiment. The care and handling was according to international guidelines for the use and maintenance of experimental animals.[17]

Analgesic Activity (Writhing Test)

The acetic acid induced writhing method is an analgesic behavioral observation assessment method that demonstrate a noxious stimulation in experimental animal.[18] The experiment was done by following Nguemfo et al. with little modification.[19] The mice were divided into 6 groups, each of containing six mice and denoted as control (distilled water), standard (diclofenac), pet ether 200, pet ether 400, chloroform 200, and chloroform 400. Mice were fasted for 18 hr prior to experiment and fed the treatment post orally. The dose of treatment were as like follows- DW 10 ml/kgbw, diclofenac 100 mg/kgbw, pet ether extract 200 mg/kgbw, pet ether extract 400 mg/kgbw, chloroform extract 200 mg/kgbw, and chloroform extract 400 mg/kgbw. After 1 hr of post oral treatment of control, standard, and plant extract, 0.6% of glacial acetic acid was introduced intraperitoneally. The writhing was observed for 15 minutes and the percentage of analgesic activity was calculated as follows.[19]

Antioxidant Activity

Reducing Power Assay

The reducing power assay is based on the principle of increase of absorbance of supplied sample present in mixture. The higher the absorbance value indicates higher the reducing power.[20] The experiment was done by following published article with little modification.[21,22] The 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of $K_3Fe(CN)_6$ (1% w/v) were added to various concentration (40-500 µl/mL) of plant extract dissolved in methanol. The mixture was incubated at 50°C for 20 min, followed by addition of 2.5 mL of trichloroacetic acid (10% w/v). Then the mixture was centrifuged at 3000 rpm for 10 min to collect the supernatant (2.5 mL). The supernatant was mixed with distilled water (2.5 mL) and 0.5 mL of $FeCl_3$ (0.1%, w/v). The absorbance was measured at 700 nm against blank

sample. Higher absorbance value indicates stronger reducing power. The antioxidant activity of plant extract was expressed as half maximal concentration of effective concentration (EC_{50}) and the EC_{50} value was calculated by equation (Y = mX + C; When Y = 0.5, X = EC_{50}) obtained from the graph [Figure 2].[23]

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay

The antioxidant activity of organic solvent soluble fractions of plant extract was determined using DPPH free radical scavenging assay following published article with some modification.[24-26] An aliquot of 300 µl of different concentrations (20-500 µg/ml) of each of the extract was mixed with 3 ml of freshly prepared DPPH solution (0.1 mM) in methanol. The reaction mixture was mixed and incubated for 30 min at room temperature in dark and its absorbance was taken at 517 nm. Ascorbic acid was used as the reference standard. The DPPH scavenging capability was calculated using the following equation-

Where, Abs (control) is the absorbance of the DPPH radical in methanol; Abs (sample) is the absorbance of the DPPH solution in presence of the respective solvent soluble (pet ether and chloroform) extract or standard. The antioxidant activity was expressed as IC_{50}, and the IC_{50} was calculated by the equation (Y = mX + C; when Y = 50, X = IC_{50}) obtained from the graph [Figure 2].

Ferric Reducing Antioxidant Power Assay

The reducing potential of plant extract was determined by following the method described by Chen and Khantaphant with little modification.[27,28] The FRAP reagent was freshly prepared by mixing of 100 mL acetate buffer (300 mM, pH 3.6), 10 mL of TPTZ solution (10 mM TPTZ in 40 mL Hydrochloric acid) and 10 mL of ferric chloride heptahydrate (20mM). The solution was warmed at 37 °C for 30 min under dark condition. The FRAP reagent (3 mL) was then added to 0.3 mL of various concentration of plant extract or absolute methanol (blank). The mixture was incubated for 5 min in dark and the absorbance was measured at 593 nm. The antioxidant potential (EC_{50}) of the sample was calculated from a linear regression analysis [Figure 2] and compared with standard of ascorbic acid.

Statistical Analysis

The results were presented as mean ± standard error of mean (SEM). The one-way ANOVA with Dunnett's post hoc test was used to analyze and compare the data using GraphPad Prism version-5 (GraphPad Software, San Diego California USA)., while < 0.05–0.001 were considered statistically significant value.

RESULTS

The whole plant extract of *A. ilicifolius* was tested to check the analgesic activity in mice model. The pet ether and chloroform extract was evaluated for possible analgesic activity by 0.6% acetic acid

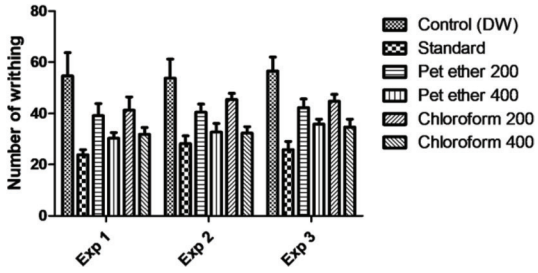

Figure 1 Number of writhing in Swiss albino mice after post oral administration of DW, plant extract, and standard diclofenac. Pain was induced by peritoneal administration of 0.6% acetic acid and writhing was counted up to 15 minutes. Results were expressed in Mean ± SEM of six mice in each group and three independent experimental results were shown

Table 1 **Analgesic activity of whole plant extract of *A. ilicifolius* on Swiss albino mice**

Group	Treatment	Number of writhing (Mean ± SEM)	% inhibition of analgesic activity
I	DW (10 ml/kgbw)	55 ± 4.03	—
II	Standard Diclofenac (100 mg/kgbw)	25.94 ± 1.58***	52.79 ± 2.62
III	Pet Ether extract (200 mg/kgbw)	40.67 ± 1.95***	26.06 ± 1.14
IV	Pet Ether extract (400 mg/kgbw)	32.94 ± 1.5***	40.14 ± 2.32
V	Chloroform extract (200 mg/kgbw)	43.89 ± 2.01*	20.79 ± 4.12
VI	Chloroform extract (400 mg/kgbw)	32.94 ± 1.53***	40.12 ± 0.90

Results are expressed in Mean ± SEM of three independent experiment and $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$ were considered as statistically significant value.

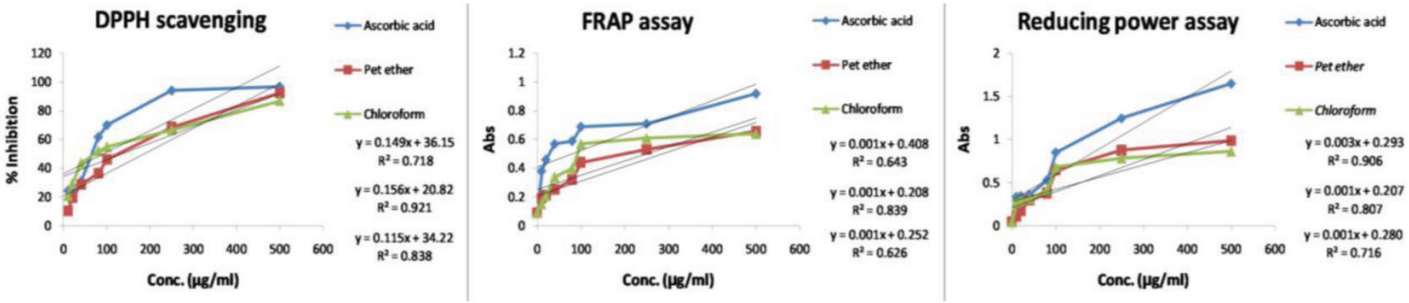

Figure 2 DPPH scavenging activity, ferric reducing antioxidant power assay, and reducing power assay of standard ascorbic acid, pet ether and chloroform soluble fractions of whole plant extract of *A. ilicifolius*

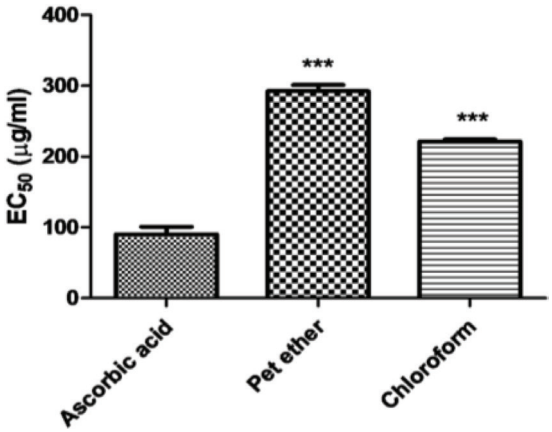

Figure 3 The EC_{50} value (µg/ml) of ascorbic acid, pet ether, and chloroform soluble fractions in reducing power assay. Results are expressed in Mean ± SEM of three independent experiment and $p^{*} < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$ were considered as statistically significant value

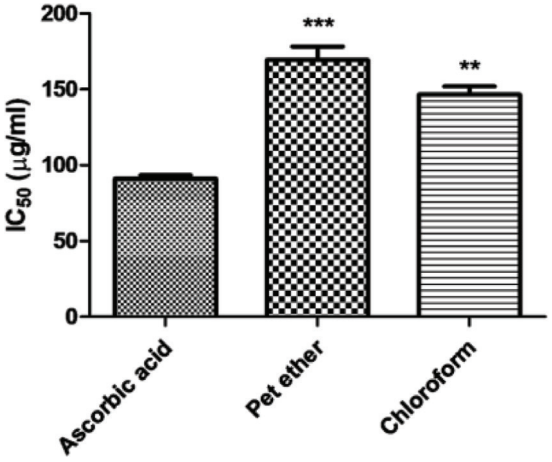

Figure 4 The IC50 value (µg/ml) of DPPH scavenging effect of ascorbic acid, pet ether, and chloroform soluble fractions. Results are expressed in Mean ± SEM of three independent experiment and $p^{*} < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$ were considered as statistically significant value

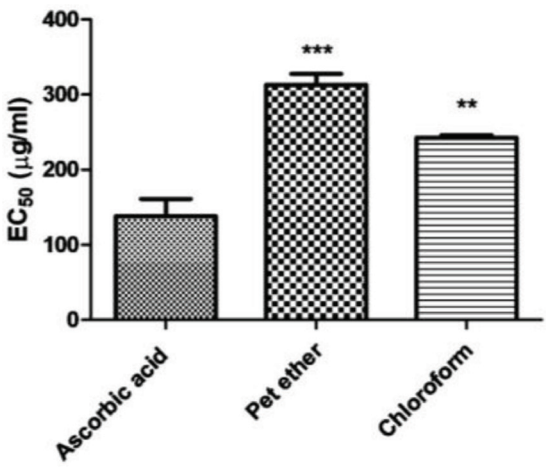

Figure 5 The EC_{50} value (µg/ml) of ferric reducing antioxidant power activity of ascorbic acid, pet ether, and chloroform soluble fractions. Results are expressed in Mean ± SEM of three independent experiment and $p^{*} < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$ were considered as statistically significant value

induced writhing method. The effect of pet ether and chloroform soluble fraction of plant extract and standard diclofenac was compared with control (DW). The writhing number in control (DW) group of mice was found to be high compare to test sample and standard drug [Figure 1].

Both of pet ether and chloroform extract was found to provide analgesic activity in a dose dependent fashion. The percent of analgesic activity was found to be (26.06 ± 1.4)% at 200 mg/kgbw and (40.14 ± 2.32)% at 400 mg/kgbw concentration [Table 1]. On the other hand chloroform extract showed (20.79 ± 4.12)% analgesic inhibitions at 200 mg/kgbw and (40.12 ± 0.9)% at 400 mg/kgbw concentration, respectively. The standard drug diclofenac revealed inhibition of analgesic activity by (52.79 ± 2.62)% at a concentration of 100 mg/kgbw [Table 1]. Compare to standard drug diclofenac, the plant extract provide moderate analgesic activity, which is reasonable.

The whole plant extracts of pet ether and chloroform fraction was also subjected to evaluate the possible antioxidant activity. The plant extracts may possess different phytochemical constituents which provided antioxidant activity.[29] In reducing power assay, the pet ether and chloroform fraction of plant extracts showed potential antioxidant activity. The absorbance for antioxidant activity was concentration dependent and showed linear increment of absorbance value with increase concentration of plant extract as like standard ascorbic acid. The EC_{50} value of pet ether and chloroform fraction were obtained (292.7 ± 8.37) µg/ml, and (221.3 ± 2.96) µg/ml, respectively [Figure 3]. The standard ascorbic acid showed EC_{50} value of (90 ± 10.89) µg/ml. From the results, it is to be mentioned that the antioxidant potential of pet ether and chloroform soluble fractions are low to moderate compare to standard ascorbic acid.

In DPPH scavenging activity assay, the plant extract was also showed antioxidant potential at a similar fashion as like reducing power assay. The percent inhibition of scavenging activity was shown to be concentration dependent and was found to be increased with plant extract concentration [figure 2]. To better understand the antioxidant activity, IC_{50} value was calculated from the graph and derived equation. The IC_{50} value of pet ether and chloroform fraction of plant extract was shown to be (169.4 ± 8.86) µg/ml and (146.6 ± 5.39) µg/ml, respectively [Figure 4]. Standard ascorbic acid revealed the IC_{50} value of (91.07 ± 2.31) µg/ml.

In FRAP assay, the plant extract showed antioxidant activity as like DPPH and reducing power assay. The absorbance was found to be increased with the concentration of plant extract up to a level, then found to be constant at higher concentrations [Figure 2]. The EC_{50} value of pet ether and chloroform fraction of plant extract was found to be (313 ± 14.57) µg/ml and (242.7 ± 3.52) µg/ml, respectively [Figure 5]; whereas, the EC_{50} value for standard ascorbic acid was found to be (138.0 ± 23.26) µg/ml [Figure 5].

DISCUSSION

Inflammation is the common sign and symptom of expressing body abnormalities by any external injuries, edema, and pathological situation in human. During the pathological situation, body response to external hit, trauma, bacterial infection or any other injury with inflammation. Conventional NSAIDs are used to treat inflammation, pain, bruise and edema. However, severe side effects and toxicity is major concern during the choice of treatment regimen. To overcome the treatment limitation and avoid adverse effects, finding of new molecular entities is prime concern of researcher. However, new molecular entities could be synthesized in synthetic laboratories; the medicinal plants are considered as a major source due to more toxic nature of synthetic drug.[5] In this study, pet ether and chloroform soluble fractions of whole plant extract of *A. ilicifolius* was evaluated for possible anti-inflammatory activity by acetic acid induced writhing in Swiss albino mice model. The results revealed that, the whole plant extracts of *A. ilicifolius* possesses significant anti-inflammatory activities [Figure 1 and Table 1].

Intraperitoneal administration of acetic acid induces capillary permeability and fluid infiltration with protein exudation. The event produces acute inflammation which is express by writhing of abdomen. Besides, during the inflammation, leukocytes and macrophage migrates to the side of injuries and produce hydrogen peroxide. Hydrogen peroxide may transform highly reactive hydroxyl radical in the presence of transitional elements. This hydroxyl radical may acts as second messenger to activate inflammatory mediators.[30] In this study, antioxidant activity by different method like DPPH scavenging activity, FRAP assay, reducing power assay revealed the significant antioxidant activity of whole plant extract of *A. ilicifolius*. These antioxidants may inhibit the generation of reactive oxygen species and pro-inflammatory mediators to prevent the generation of inflammation as like aspirin.[31] Even more, the phytochemical bioactive compounds in *A. ilicifolius* may inhibit inflammation by modulating cyclooxigenase—1 and 2 (COX—1 and COX—2), lipooxygenase (LOX), and nuclear transcription factor (NF-κB).[32,33] The plant extract is found to be decreased protein exudation and leukocyte migration in the peritoneal fluid, indicating its effectiveness towards inhibition of peritoneal inflammation.[5]

Free radicals are responsible for the generation of cancer by means of different steps. Most commonly, reactive oxygen species and nitrogen species could facilitate cancer development by damaging of DNA and other biomolecular components.[34] Chemotherapeutic drugs are the first choice of cancer treatment; however they can trigger enormous side effects. Considering this drawback, research on phytochemical screening and discovering anticancer drug is becoming attractive to the researcher day by day. The phytochemical components in medicinal plants are believed to inhibit carcinogenesis and several studies showed that the antioxidant compounds exert cytotoxic effect on tumor cells and in *in—vivo* animal model.[35-37] Thus, the possible antioxidant potential of pet ether and

chloroform soluble fraction of whole plant extract of *A. ilicifolius* was evaluated.

In antioxidant test, the DPPH scavenging activity assay showed DPPH scavenging with the increasing concentration of plant extract [Figure 2]. The IC_{50} value of pet ether and chloroform fraction was found to be fair proximity of standard ascorbic acid. Result of free radical scavenging activity can be varied depending on several factors such as, stereo selectivity of the radicals, solubility of the extract in different solvent systems, polarity of solvent, and functional group present in bioactive compounds. The results of this study suggesting possible antioxidant activity of plant extract which may use in the treatment of free radical induced pathological damages.[38]

In FRAP assay, it has been shown that the absorbance is increased steadily with the increase of plant extract concentration indicating potential antioxidant activity. The EC_{50} value of pet ether and chloroform fraction was found to be higher, however it was near to standard ascorbic acid value. In reducing power assay, both of pet ether and chloroform fraction showed moderate antioxidant activity as like DPPH and FRAP assay. The EC_{50} value of pet ether and chloroform soluble fractions was found to be (313.0 ± 14.57) µg/ml and (242.7 ± 3.52) µg/ml, respectively. Standard ascorbic acid showed the EC_{50} value of (138.0 ± 23.26) µg/ml. From the results, it has been implied that, the plant extract possesses moderate to low ferric reducing capability.

In conclusion, the pet ether and chloroform soluble fraction of whole plant extract of *A. ilicifolius* showed potential analgesic and antioxidant activities. However, it is highly necessary to isolate the single compound and to evaluate the mentioned activities by single compound for better understanding of plant medicinal value.

CONFLICT OF INTEREST

The author declared they have no competing interest.

FINANCIAL DISCLOSURE

The authors declared that this study received no financial support.

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