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Research Article

Chemical Constituents Isolated from the Leaf Extracts of *Amomum aculeatum* Roxb. (Zingiberaceae), *In Vitro* Evaluation of their Antioxidant, Antimicrobial and Anti-inflammatory Activities

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ABSTRACT

Members of the genus *Amomum* are well known for their biological activities. In the present study, chemical constituents, antioxidant, antimicrobial and anti-inflammatory activities with total phenols and total flavonoids from the leaf extract of *Amomum aculeatum*, belongs to the family Zingiberaceae were analysed. Chemical constituents screening was carried out following the standard protocols. Antibacterial activity was assayed by a well diffusion method using Muller Hilton agar media. Antifungal activity was analyzed by a well diffusion method using potato dextrose agar. Radical scavenging activity was evaluated by ABTS, DPPH and reducing power assay. Total phenols and flavonoids were estimated by waters 510 HPLC. The anti-inflammatory evaluation was conducted by inhibition of protein denaturation method. Chemical constituents screening showed the presence of tannins, flavonoids, cardiac glycosides, terpenoids, carbohydrates, proteins and total phenols. Antioxidant activity recorded a significant difference in DPPH and reducing power assay compare to ABTS method. Ethanol and aqueous leaf extracts displayed significant anti-inflammatory activity compared to standard (Aspirin). Study recommends the prospect of *Amomum aculeatum* leaf used both as a medicine and spices as was shown to have significant antioxidant, antimicrobial and anti-inflammatory activities.

Keywords: *Amomum aculeatum*; Antimicrobial; Anti-inflammatory; Antioxidants; ABTS; Chemical constituents; DPPH; HPLC; Zingiberaceae

INTRODUCTION

Plants are considered to have a rich source of naturally occurring bioactive compounds with high medicinal properties against various diseases. Diseases that remain most challenging for today's health care system tend to be more complex than could be treated by current combination therapies. However, plant based drugs contain a mixture of multiple components which serve the effective control of disease [1]. The use of medicinal plants for local remedies is a traditional custom, and as we know medicinal plants have the potency to combat all most all diseases. Plants belonging to Zingiberaceae family are commonly known as ginger and it is one of the major medicinal herbs which have strong aromatic and properties. Variety of Zingiberaceae species are indigenous to tropics dispersed extensively all over the tropics, predominantly in South Asia and Southeast Asia represented by almost 50 genera and 1300 species [2]. The species are commonly distributed in India, tropical Asian and extended to northern Australia [3]. Zingiberaceae family is a significant natural reserve throughout the world since the emergence of mankind that offers numerous beneficial properties for aesthetics, dyes, food, medicines, perfume and spices [4]. *Amomum* is one of the largest genus in the ginger family Zingiberaceae and broadly distributed

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throughout the globe [5-7]. Genus *Amomum* belongs to the division of spermatophyte, the sub-division of angiosperm, the class of monocotyledons, the order of Zingiberales, the family of Zingiberaceae, the sub family of Zingiberoideae and the tribe of Alpineae. This genus has around 90-150 species, where 40-50 of them grows in Asia [8-11]. In the North East region of India, 19 genera and around 88 species are reported [12]. Genus *Amomum* (Zingiberaceae) is profitably used as flavour spices, perfume, medicinal stuff, garnishing plants. Their fruits contain high amount of volatile oil and the chemistry of volatile oil is one of the most prospective characters for taxonomy [13].

In India, eight species of genus *Amomum* were identified as an important source of traditional medicine [14]. According to the recent report, different species of *Amomum* were found to be a used as a remedy for malaria, nausea, stomach disorders, cancer and inflammation [15-16]. The genus *Amomum* is mainly distributed in the Northeastern states, Sikkim, Peninsular and Andaman Nicobar Island, are generally evergreen perennial herb, inhabiting wet forest margins, and different species are widely used as spices, vegetable and in medicine due to its therapeutic potential [17-19]. *Amomum subulatum* used as a traditional drug in ayurvedic pharmacopoeia due to its curative and preventive properties for various ailments. The major constituent of *Amomum subulatum* essential oil is 1, 8-cineole, monoterpene hydrocarbon and terpinols. The monoterpene hydrocarbon content is in the range of 5%-17% of which lamonene, sabeinene and pinenes are present ominously. Terpinols comprise roughly 5%-7% of the oil. Due to the presence of these compounds, it has pharmacognostic properties such as analgesic, antimicrobial, cardiac stimulant, carminative, diuretic, stomachic etc. [20]. *Amomum aculeatum* Roxb is a tall herb with leafy stem, distributed in India, Myanmar, Thailand, Malay Peninsula and Indonesia. These are tropical rain or dry evergreen forest plants grown in a shaded place. Plants are characterized by pubescent leaf, and many long inflorescences at the base with many pedicelled yellow-pink flowers. Fruits are purple in color, covered with green spines and are edible. All parts of this plant are used in folk medicine to treat fever, pain, malaria and influenza [21]. Scientists have reported the chemical constituents and biological efficacy of some species of *Amomum* [22]. Minor compounds like 1,7-dioxa-dispiro[5.1.5.2] pentadeca-9,12-dien-11-one derivative, aculeatin D, and 5-hydroxyhexacos-1-en-3-one was isolated from the rhizome of *Amomum aculeatum* which was reported to possess antibacterial potential against *Bacillus cereus*, *Escherichia coli* and *Staphylococcus epidermidis* [23]. Chiefly owing to its inadequacy, particularly in *Amomum aculeatum* there is no scientific investigations have been reported in terms of biological activity. An exploration of these constraints encompasses the uniqueness of this work. Therefore, in this study, an attempt has been made to evaluate the phytochemicals, radical scavenging potential, antimicrobial and anti-inflammatory activity with total flavonoids and total phenols by HPLC was assessed in leaf extracts of *Amomum aculeatum*.

MATERIALS AND METHODS

Collection and identification of plant material

The fresh leaves of *Amomum aculeatum* were collected from Andaman and Nicobar Island and taxonomic identification of the plant was confirmed by Plant Taxonomy Professor, Department of Botany, Bangalore University, Bengaluru, Karnataka, India.

Extraction of plant material

Amomum aculeatum plant leaves were first surface sterilized with distilled water and then dried at 45°C for 3-4 days. Dried leaves were made into fine powder by using a grinder and sieve 10 micron mesh. About 50 gm of fine powder was used for successive solvent extraction, such as petroleum ether, ethyl acetate, chloroform, ethanol and water by using soxhlet apparatus. Extracts were condensed by the rotary evaporator and crude extract obtained was stored at 4°C in airtight containers for further analysis. For water extract, 20 g of powdered leaf sample was kept in water bath for 8 hrs at 40°C. Extract was filtered, centrifuged, concentrated and stored at 4°C until use. All the extracts were dissolved in respective solvents prior to analysis and subjected to quantitative determination of primary and secondary metabolites [24-25].

Phytochemical screening

Phytochemical screening for the crude leaf extracts of *Amomum aculeatum* were subjected to preliminary phytochemical screening to determine the presence of tannins, alkaloids, diterpenes, flavonoids, cardiac glycosides, terpenoids, steroids, carbohydrates, proteins and phenols [26-27].

Determination of total phenols and total flavonoids by HPLC

Total phenols and total flavonoids were analyzed by waters 510 HPLC. The mobile phase included acetonitrile and water (70:30) with flow rate 1 ml/min, and 20 µl injection volume. The column used was C18 and 1200 psi pressure was maintained. Phenol compounds were detected by UV absorption at 254 nm with gallic acid as standard whereas, quercetin was used as standard for flavonoids and absorbency was detected at 272 nm [28,29]. Phytochemicals were recognized based on retention time and quantification was done by using area.

Antioxidant activity

Radical scavenging activity of different solvent extracts of *Amomum aculeatum* leaf was carried out by ABTS, DPPH and reducing power assay methods. All the assays were carried out in triplicates.

ABTS radical scavenging assay

Free radical scavenging ability of leaf extracts was determined by ABTS radical cation decolourization assay procedure [30]. $ABTS^{+}$

cation radical was produced by the reaction between 7 mm ABTS in water and 2.45 mm potassium persulfate (1:1), stored in dark at room temperature for 12 hour-16 hour before use. $ABTS^+$ solution was then diluted with methanol to obtain an absorbance of 1.0 at 734 nm. Different concentrations of leaf extracts were added to 3.0 ml $ABTS^+$ solution, the absorbance was measured at 734 nm after 30 min of initial mixing by spectrophotometer. An appropriate solvent blank was run with each assay. Percent inhibition of absorbance was calculated using the formula,

$ABTS^+$ Scavenging effect (%)=$(A_B–A_A)/AB) \times 100$

Where,

A_B: Absorbance of ABTS radical+methanol.

A_A: Absorbance of ABTS radical+sample extract/standard.

BHT: Used as standard substance.

DPPH radical scavenging assay

Free radical scavenging activity of leaf extracts was screened by assessing their ability to discolor DPPH (2,4-dinitrophenyl-1-picrylhydrazyl) in methanol. Radical solution is prepared by dissolving 24 mg DPPH in 100 ml methanol [31]. Different aliquots of leaf extracts were taken; 3 ml of DPPH solution was added and incubated in dark conditions for 30 min. Finally the absorbance of each aliquot was measured at 517 nm against methanol as blank by UV spectrophotometer. A calibration curve was plotted with percentage DPPH scavenged versus standard antioxidant concentration (BHT).

Reducing power assay

Reducing power assay was measured according to the procedure described [32]. Various concentrations of leaf extracts were taken in test tubes, 2.5 ml of 200 mm sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide were added. The mixture was incubated at 50°C for 20 min. After that 2.5 ml of 10% trichloroacetic acid (w/v) was added, then the mixture was centrifuged at 650 rpm for 10 min. Later, 5 ml aqueous layer was mixed with 5 ml deionised water and 1 ml of 0.1% ferric chloride, then absorbance was measured at 700 nm. A calibration curve was plotted with reducing power against BHT as standard.

Antimicrobial assay by well diffusion method

The minimum inhibition concentration was determined according to procedure defined in broth microdilution method using 96 well micro titer plates [33]. Leaf extracts of were subjected to antimicrobial assay by using the agar well diffusion method. Pure cultures of pathogenic gram positive (*Staphylococcus aureus* and *Bacillus cereus*), gram negative (*Escherichia coli, Proteus vulgaris* and *Klebsiella pneumonia*) bacterial strains, obtained from MTCC and NCIM and maintained in the Muller Hinton agar medium. Fungal cultures such as *Aspergillus niger, Aspergillus flaovus, Candida albicans and Cryptococcal* sp., preserved in potato dextrose agar. Inhibition zone was measured and recorded. Tetracycline was used as positive control.

Anti-inflammatory activity by inhibition of protein denaturation assay

Anti-inflammatory activity of different extracts of *Amomum aculeatum* leaf was evaluated by inhibition of protein denaturation assay [34]. Incubate the reaction mixture including 1 ml different aliquots of plant extracts and 1% solution of bovine albumin at 37°C in a water bath for 20 min. The absorbance of the reaction mixture was measured at 680 nm after cooling using spectrophotometer. Aspirin was used as standard. The percentage of inhibition of protein denaturation was calculated as $(A_B–A_A)/A_B) \times 100$

Statistical analysis

Statistical facts were uttered as mean ± Standard Error of Mean (SEM) (n=3). Linear regression was executed to conclude 50% Inhibitory Concentration (IC_{50}). The difference among the experimental and control groups was determined using GraphPad Prism® version 7.0, the comparison carried out by One-Way Analysis of Variance (ANOVA). The substantial difference in the experimental groups was evaluated by the Least Significant Difference (LSD) post hoc analysis to test the significance at $P<0.05$.

RESULTS

Preliminary phytochemical screening of *Amomum aculeatum* leaf extract in different solvents such as petrol ether, chloroform, ethyl acetate, ethanol and aqueous were tested for the presence of primary and secondary metabolites. Result revealed the presence of tannins, alkaloids, diterpenes, flavonoids, cardiac glycosides, terpenoids, steroids, carbohydrates and proteins (Table 1). Cardiac glycosides present in all the extracts except in petroleum ether. Tannins, flavonoids, carbohydrates and phenols were present whereas, terpenoids were absent in ethanol and aqueous extracts, and proteins were found in aqueous extract. Alkaloids, steroids and diterpenes were not reported in any solvent extracts.

Table 1: Phytochemical screening in different solvents extracts of *Amomum aculetum* leaf.

Phytochemicals	Test	Petroleum ether	Chloroform	Ethyl acetate	Ethanol	Water
Tannins	$Fecl_3$	-	-	-	+	+
Alkaloids	Wagners	-	-	-	-	-
Diterpenes	Copper acetate	-	-	-	-	-
Flavonoids	Lead acetate	-	-	-	+	+
Cardiac glycosides	Keller-killiani	-	+	+	+	+
Terpenoids	Salkowski	+	+	+	-	-
Steroids	Leiberman	-	-	-	-	-
Carbohydrates	Molisch	-	-	-	+	+
Proteins	Xanthoproteic	-	-	-	-	+
Phenols	FC Method	-	-	-	+	+

HPLC results reveal the presence of phenols compare with gallic acid and their retention time was 2.25 min and flavonoids compare with quercetin and their retention time was 2.2 min. Concentration was calculated using the area and the amount of phenols present in ethanol extract was (1.61 mg/gm) and aqueous extract was (0.72 mg/gm) (Figure 1). The concentration of flavonoids present in ethanol extract was 1.92 mg/gm and aqueous extract was 1.02 mg/gm (Figure 2). Furthermore, in ABTS method, ethanol (40%-66.8%) and aqueous (41.2%-62.0 %) extracts of *Amomum aculeatum* leaves have recorded the maximum radical scavenging activity that is equivalent to the standard ascorbic acid. Petroleum ether, chloroform and ethyl acetate extracts have been reported minimum activity. The reducing antioxidant activity of *Amomum aculeatum* leaves revealed the highest activity in ethyl acetate, ethanol and aqueous with maximum of 42.7%, 48.8% and 48.3% respectively compared to petroleum ether (21.8%) and chloroform (28.22%) at the concentration of 500 µg/ml. The result was expressed as ascorbic acid equivalent antioxidant capacity.

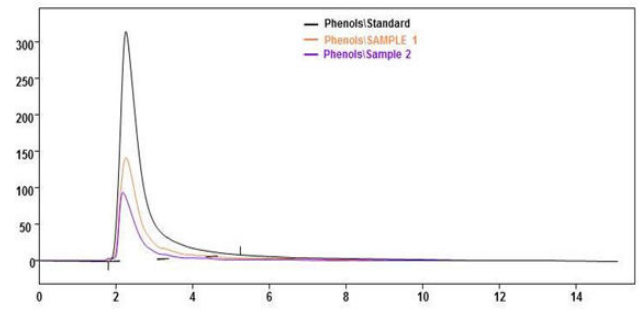

Figure 1: Chromatographic profile of phenol content.

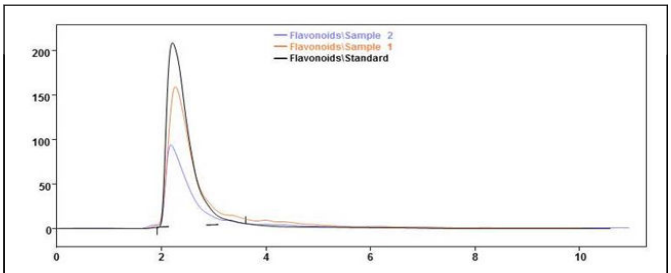

Figure 2: Chromatographic profile of flavonoid content.

Antioxidant activity

In vitro antioxidant potential of different solvent extract of *Amomum aculeatum* leaf extract was investigated by DPPH and ABTS method. According to the obtained result, leaf extract of petroleum ether, chloroform and ethyl acetate showed minimum to moderate radical scavenging efficacy with 1.2%-14.3%, 7.9%-34.7% and 31.1%-64% respectively. Whereas, ethanol (43.7%-77.5%) and aqueous extract (40.6%-73%) was reported a significant activity, comparable with standard at the concentration of 50-500 µg/ml (Figures 3-5).

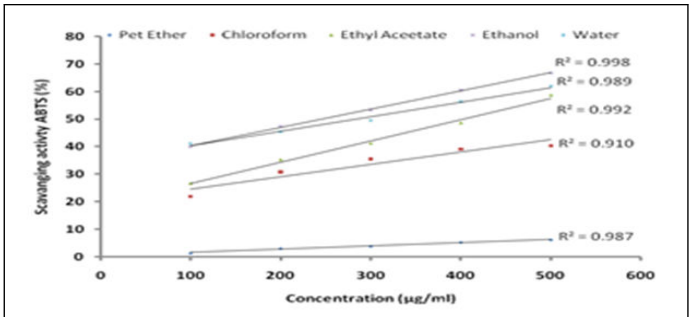

Figure 3: ABTS free radical scavenging activity of different solvents of *Amomum aculetum* leaf extracts.

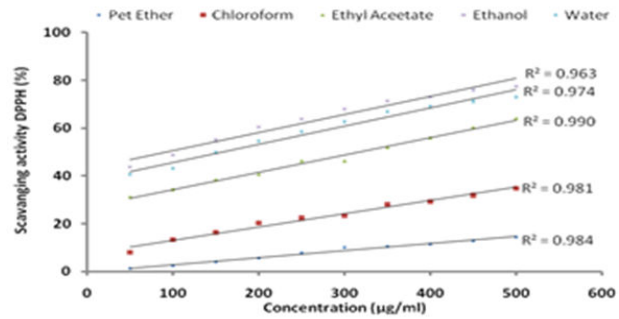

Figure 4: DPPH free radical scavenging activity of different solvents of *Amomum aculetum* leaf extracts.

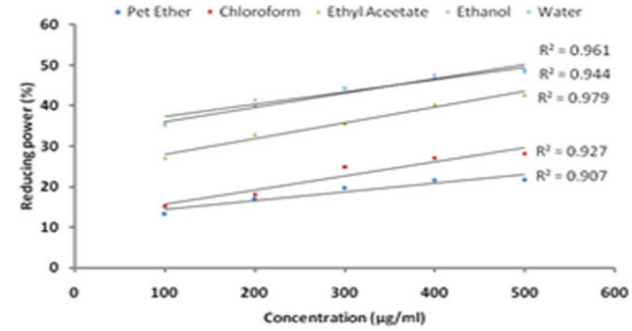

Figure 5: Reducing power assay of different solvents of *Amomum aculetum* leaf extracts.

Antimicrobial activity

According to the present assay, gram negative bacteria *E.coli* were inhibited by all the extracts except chloroform. *K. pneumonia* was inhibited by ethanol and ethyl acetate extract, whereas *Proteus vulgaris* was inhibited by ethanol and water extracts. Gram positive bacteria such as *B. cereus* inhibited by all the extracts except chloroform. Ethanol and water extracts inhibited *S. aureus* (Table 2). Antifungal activity revealed that, *A. niger* was inhibited by petroleum ether and ethanol extracts (Table 3). *A. flaovus* inhibited by ethyl acetate, ethanol and water extracts whereas *C. albicans* was inhibited by petroleum ether, ethyl acetate and water extracts but *Cryptocaccal* sp. was not inhibited by any of the tested extracts. Different concentrations (50 µl/mL-150 µl/mL) of extracts were used to find minimal inhibition concentration zone of inhibition using well diffusion method.

Table 2: Antibacterial activity of different solvents extracts of *Amomum aculetum* leaf.

Plant extracts	Volume (µl)	*E. coli*	*S. aureus*	*P. vulgaris*	*K. pneumoniae*	*B. cereus*
Petroleum ether	50	-	-	-	-	-
	100	-	-	-	-	-
	150	12	-	-	-	11
Chloroform	50	-	-	-	-	-
	100	-	-	-	-	-
	150	-	-	-	-	-
Ethyl acetate	50	-	-	-	-	-
	100	-	-	-	-	-
	150	11	-	-	7	10
Ethanol	50	-	-	-	-	-
	100	-	-	-	7	-
	150	10	13	12	8	-
Water	50	-	-	-	-	-
	100	-	-	12	-	9
	150	11	15	13	-	12
Positive control Tetracycline (30 µg)		-	-	-	-	-

Table 3: Antifungal activity of different solvents extracts of *Amomum aculetum* leaf.

Plant extracts	Volume (µl)	*Crytococcus*	*Candida albicans*	*Aspergillus niger*	*Aspergillus flavus*
		Zone (mm)			
Petroleum ether	50	-	-	-	-
	100	-	-	-	-
	150	-	7	8	-
Chloroform	50	-	-	-	-
	100	-	-	-	-
	150	-	-	-	-
Ethyl acetate	50	-	-	-	-
	100	-	-	-	-
	150	-	8	-	7
Ethanol	50	-	-	-	-
	100	-	-	6	-
	150	-	6	9	7
Water	50	-	-	-	-
	100	-	-	-	-
	150	-	-	-	9
Positive control Fluconazal (30 µg)		-	-	-	-

Anti-inflammatory activity

In the present study, a significant inhibition of albumin denaturation was observed in both ethanol and aqueous leaf extract of *Amomum aculeatum* and the estimated IC_{50} value for the ethanol and aqueous fractions were 445.73 µg/mL and 480.44 µg/mL respectively compared to standard aspirin. Rate of inhibition of protein denaturation increased with the increase in concentration of studied plant extract (Figure 6).

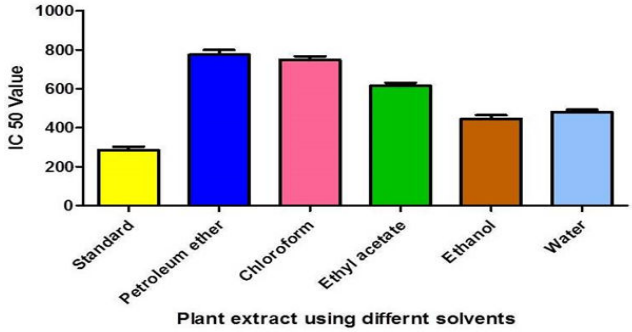

Figure 6: Protein denaturation assay of different solvents extracts of *Amomum aculetum* compared with the standard drug aspirin.

DISCUSSION

Plants produce a number of chemicals for several resolves; have medicinal and therapeutic possessions in human beings. Exploration of chemical constituents in plants is beneficial in the development of new drug and also production of several multifaceted chemicals for therapeutic use. Plants produce numerous chemicals for nutrition, defense against stress, to fight unwanted predators, and assist pollination. The diverse chemicals manufactured by plants are beneficial for human health since reported to produce several useful effects on humans owing to their medicinal possessions [35]. The plants generally comprise several chemical constituents comprising alkaloids, tannins, flavonoids, phenolics and other secondary metabolites, have been testified to stimulate physiological and biochemical reaction in humans [36]. According to the previous reports, different species of *Amomum* exhibits antioxidants, antimicrobial and anti-inflammatory efficiency [37-39]. Present results revealed the presence of several chemical constituents like phenols, tannins, flavonoids, carbohydrates, cardiac glycosides, terpenoids and proteins from different solvent extracts of *Amomum aculeatum* leaf. These chemicals play vital role in preventing disease in human beings. The concentration of active compounds like phenols and flavonoids of medicinal plants, depending on the place, topography and season of collection [40-42].

Advanced studies are needed for this plant to find therapeutic property. Free radicals are highly reactive and causing the damage to cell by increasing superoxide and decreasing nitric oxide formation [43,44]. Antioxidant activity is due to different reactions like free radical scavenging activity, reducing power capacity and oxygen radical absorbing capacity which prevents the damage to cell by neutralizing the electron [45-47]. Free radical scavenging activity such as DPPH, ABTS and reducing power methods are extensively used because DPPH and ABTS is dissolved readily in organic solvents. The antioxidant analysis in ethanol leaf extract of *Amomum aculeatum* showed moderate to highest activity in DPPH followed by ABTS and reducing power assay with IC_{50} value of 93.2 µg/ml, 247 µg/ml and 320 µg/ml respectively. Petroleum ether extract reported to have significantly low activity in all the three methods. Microorganisms survived for several years because of their ability to adapt antibiotics [48]. The rate of microbial resistances for accessible therapeutic agents poses a serious medical problem to overcome the use of relevant phytochemicals. There is a lack of standard protocols for the use of phytochemicals to treat diseases by traditional medical practitioners [49-50].

In the present study, ethanolic leaf extract was found active against *Escherichia coli, Staphylococcus aureus, Proteus vulgaris, Klebsiella pneumonia, Bacillus cereus,* and fungus like *Aspergillus niger, Aspergillus flaovus and Candida albicans*. In aqueous extract, antibacterial activity was significantly more but did not show any activity in fungal species except in *Aspergillus flaovus*. Ethanol extracts certainly much better and powerful compare to aqueous extract and this may be due to better solubility of the active components in organic solvents [51-53]. Inflammation occurs when protein does not function biologically due to injuries, infection or by a defective immune system [54,55]. A variety of medicinal plants with anti-inflammatory potential can ease the inflammation with no side effects [56-59]. Recently, scientists have evaluated some species of genus *Amomum* (Zingiberaceae) and reported, whereas, *Amomum aculeatum* in particular has not been documented [60-63]. According to the present result, ethanol and aqueous extract of *Amomum aculeatum* leaf reported a promising activity that concentration dependent inhibition of protein denaturation.

CONCLUSION

These results suggest that the bioactive constituents of *Amomum aculeatum* have antioxidant, antimicrobial and anti-inflammatory activities. In comparison, ethanolic and aqueous extract *Amomum aculeatum* leaf reported a significant antioxidant and anti-inflammatory potential, whereas petroleum ether, chloroform and ethyl acetate possess minimum to moderate activity. Furthermore the present result offers a precise scientific data about the biological properties of the studied plant. Advance research is necessary to identify and characterize the active compounds involved in biological activity.

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