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Antioxidant activity of ethanolic extract of *Alpinia calcarata* Rosc. Rhizome

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Abstract

Alpinia calcarata belonging to the family of Zingiberaceae is commonly found in Bangladesh and has been conventionally used in medicine systems for the prevention of many diseased conditions. The present study was conducted to explore the antioxidant activity of ethanolic extract of *Alpinia calcarata* rhizome (EEACR). EEACR was studied for the quantitative estimation of phenolic, flavonoid, proanthocyanidin and flavonol compounds. Antioxidant activity was evaluated by using several *in vitro* standard methods. EEACR was subjected to GC-MS analysis to identify its phytoconstituents. EEACR contained a good amount of phenol, flavonoid, proanthocyanidin and flavonol. The IC₅₀ value of the ethanolic extract in the DPPH, ABTS, nitric oxide, lipid peroxidation inhibition and ferrous chelating assay were 6.044, 5.266, 25.13, 21.88, and 63.33µg/ml, respectively. EEACR also showed remarkable ferric reducing and total antioxidant capacity. The total phenolic and flavonoid contents of EEACR were positively correlated (p<0.05) with DPPH, ABTS, nitric oxide, lipid peroxidation inhibition and ferrous chelating assay. In addition, the active compound present in the ethanol extract was identified as α -asarone by GC-MS analysis. In summary, our results suggest that ethanol extract of *Alpinia calcarata* rhizome possess a wide range of pharmacologically important phytochemicals which exhibited strong antioxidant activity.

Keywords: *Alpinia calcarata*, rhizome, phytochemical, antioxidant, GC-MS analysis.

1. Introduction

Oxidative stress, an imbalance between formation and neutralization of Reactive Oxygen Species (ROS), is believed to damage nucleic acids, proteins and lipids and can initiate various chronic diseases like atherosclerosis, cancer, diabetes, cardiovascular disease, ageing and inflammatory diseases [1]. Several synthetic antioxidants, such as butylated hydroxyl toluene and butylated hydroxyl anisole are largely included in human diets which are suspected to possess harmful effects on health [2]. For these reason, there is a need to find alternative and natural drugs having less or no side effects to use for oxidative stress and chronic inflammatory disease. Due to safety, availability, economical benefits and pharmacological properties such as antioxidant, anti-inflammatory activities interest is shown on plants. Plant phenolics and flavonoids are secondary metabolites with known antioxidant properties, including inhibition of oxidative enzymes, scavenging of free radicals, and induction of anti-inflammatory actions [3-4].

Alpinia calcarata Roscoe, a member of Zingiberaceae family, is widely distributed in Sri Lanka, India, and Malaysia and traditionally used in medicinal systems in Sri Lanka [5]. Rhizome is the most important part of this plant and is used to treat cough, respiratory ailments, bronchitis asthma, arthritis and diabetes [6-9]. Several studies conducted on this part have reported important biological properties including antibacterial, antifungal, antihelminthic, anti-inflammatory, antinociceptive, aphrodisiac, gastroprotective, and antidiabetic activities [10-17]. A study previously conducted by our group using Ehrlich ascites carcinoma (EAC) tumor bearing Swiss Albino mice has disclosed the cytotoxic properties of ethanolic extract of *Alpinia calcarata* rhizome [18]. Data on antioxidant properties of this plant is insufficient. Therefore, the present study investigated the phytoconstituents of ethanol extract of *Alpinia calcarata* rhizome. The study also analyzed the chemical composition of ethanol extract of *Alpinia calcarata* rhizome.

2. Material and methods**2.1 Collection of plant materials and authentication**

Rhizome of *Alpinia calcarata* Rosc (Family: Zingiberaceae) were collected from Rajshahi University area, Rajshahi, Bangladesh and were authenticated by a taxonomist at the

Department of Botany, University of Rajshahi, where a voucher specimen (No. 02) of this collection was deposited.

2.2 Preparation of extract

The dried powdered Rhizome of *Alpinia calcarata* Rosc was extracted with ethanol at room temperature and after filtration, filtrates were evaporated under reduced pressure at 40°C using a rotary evaporator to have ethanol extract.

2.3 Chemicals and reagents

2,2-diphenyl-1-picryl-hydrazyl (DPPH), potassium ferricyanide, potassium acetate, phosphate buffer, catechin (CA), gallic acid (GA), ascorbic acid (AA), AlCl₃, Trichloro acetic acid (TCA), sodium phosphate, ammonium molybdate, quercetin (QU), EDTA, methanol, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Hydroxylamine hydrochloride, N-(1-naphthyl) ethylenediamine dihydrochloride, Ascorbic acid, FeCl₃ were purchased from sigma- Aldrich, Germany.

2.4 Determination of total phenolic

Estimation total phenolics in plant extract were measured by the Folin-Ciocalteu method [19]. 0.5 ml of extract, 2.5 ml of FCR and 2.5 ml of sodium carbonate (7.5%) was added and kept for 30 min. Absorbance was recorded at 760 nm. Total phenolic contents of the extract was calculated and expressed in terms of gallic acid equivalent ($y = 0.117x + 0.051$, $R^2 = 0.998$).

2.5 Estimation of total flavonoid

Total flavonoid content in EEACR was measured by the aluminium chloride method [20]. A mixture was obtained by taking 1 ml of extracts, 5 ml of distilled water and 0.3 ml of 5% NaNO₂ in a test tube and allowed to stand for 5 minutes. After incubation, 600 µl of 10% aluminum chloride was added and allowed to stand for 5 minutes and 2 ml 1M NaOH was again added. Absorbance was recorded at 510 nm. Results were calculated and expressed in terms of catechin equivalent ($y = 0.005x + 0.047$, $R^2 = 0.998$).

2.6 Determination of total proanthocyanidin

The procedure reported by Sun *et al.* was used to determine the content of proanthocyanidin [21]. Briefly, 0.5 ml of different concentration of extracts solution was mixed with 3 mL of 4% vanillin-methanol solution and 1.5 ml hydrochloric acid and allowed to stand for 15 minutes. The absorbance was recorded at 500 nm. The results were expressed as catechin equivalent ($y = 0.002x + 0.000$, $R^2 = 0.999$).

2.7 Determination of total flavonol

The method described by Kumaran and Karunakaran was employed to estimate the total flavonol contents of the extract [22]. 2.0 ml of extract solution, 2.0 ml of 2% AlCl₃ ethanol and 3.0 ml (50 g/L) sodium acetate solutions were mixed. The mixture was kept at 20°C for 2.5 hours and the absorbance was measured at 400 nm. Using the standard curve of quercetin the flavonol contents were calculated and expressed as quercetin equivalent ($y = 0.209x + 0.010$, $R^2 = 0.997$).

2.8 Determination of total antioxidant capacity

The total antioxidant capacity of extract/standard was evaluated by Prieto *et al.*, (1999) [23]. A reaction mixture was prepared by adding 0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate. 3 ml of the resulting mixture and 0.3 ml extract/standard at different

concentrations were taken in test tubes. The reaction was completed by incubating the test tubes at 95°C for 10 minutes. The absorbance was read at 695 nm. Catechin was served as standard.

2.9 Determination of ferric reducing antioxidant capacity

The reductive potential was measured following the method of Oyaizu, (1986) [24]. 0.25 ml samples/standard (catechin) with a variety of concentrations were taken in test tubes to which 0.625 ml of potassium buffer (0.2 M) and 0.625 ml of 1% potassium ferricyanide [K₃Fe(CN)₆] solution were added and kept for 20 minutes at 50°C. Then 0.625 ml of 10% TCA was added after incubation, followed by centrifugation at 3000 rpm for 10 minutes. After that, 1.8 ml supernatant was with-drawn to which 1.8 mL of distilled water, 0.36 ml of 0.1% FeCl₃ solution was added. The absorbance was measured at 700 nm.

2.10 Determination DPPH free radical scavenging activity

Exploration of DPPH radical scavenging activity of both the sample and standard (catechin) was performed employing the method described by Choi *et al.*, (2000) with some modification [25]. 4 ml of reaction mixture consisted of 3 ml of 0.1 mM DPPH solution and 1 ml extract/standard (catechin) at different concentrations was incubated at 37°C for half an hour which is followed by the recording of absorbance at 517 nm and the percentage scavenging activity was calculated using the following formula:

$$SA\% = [(Absorbance\ of\ control - Absorbance\ of\ sample) / Absorbance\ of\ control] \times 100$$

2.11 ABTS radical scavenging assay

The ABTS assay was done according to the method described by Cai *et al.*, (2004) [26]. 7 mM ABTS stock solution was allowed to react with 2.45 mM potassium persulfate to have ABTS radical. After keeping in the dark at room temperature for 12–16 hours, the ABTS solution was diluted with water to have an absorbance of 0.70±0.02 at 734nm. 3 ml of ABTS•+ solution was mixed with 1 ml of extract/standard at different concentrations and mixtures were kept for 6 min. Then the absorbance was taken at 734 nm. Catechin was served as standard. The percentage of inhibition was measured similar to that of DPPH assay.

2.12 Scavenging activity of nitric oxide

Determination of nitric oxide scavenging was carried out as reported by Garrat, (1964) with some modification [27]. 2 ml of 10 mM sodium nitroprusside dissolved in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of plant extract at different concentrations. Each test tube was incubated for 150 minutes at room temperature which is followed by the addition of 0.5 ml of Griess reagent [1.0 ml sulfanilic acid reagent (0.33% dissolved in 20% glacial acetic acid)] and left at room temperature for 5 min. After 1 mL of naphthylethylenediamine dichloride (0.1% w/v) was added and the mixture was incubated at room temperature for 30 min. Absorbance was measured at 546 nm. Catechin was used as positive control. The percentage of inhibition was measured similar to that of DPPH assay.

2.13 Metal chelating activity

The method reported by Yuan *et al.* was employed to examine the metal chelating activity of the sample [28]. 1 ml of sample/standard at various concentration was mixed with 1 ml FeSO₄ (0.1 mM) for 30s, then 1 ml of ferrozine (0.25 mM)

was added and allowed to stand for 10 min. The absorbance was recorded at 562 nm. EDTA was used as standard reference control. The percentage of inhibition was measured similar to that of DPPH assay.

2.14 Lipid peroxidation inhibition assay

The ability of the extract to interfere with the lipid peroxidation was examined according to the method described by Liu and Ng, (2000) with some modification [29]. Liver was obtained from rat and homogenized in buffer followed by centrifugation to have liposome. 1 ml of reaction mixture prepared by mixing 0.5 ml of supernatant, 100 μ l 10 Mm FeSO₄, 100 μ l 0.1 mM AA and 0.3ml of varying concentrations of extract/standard (catechin) was incubated for 20 minutes at 37°C followed by addition of 1 ml of 28% TCA and 1.5 ml of 1% TBA. The mixture was again heated at 100°C for 15 minutes and cooled at room temperature. Catechin was served as standard. The absorbance was read at 532 nm and the percentage of inhibition was calculated similar to that of DPPH assay.

2.15 GC-MS analysis of bioactive molecule

Separation and identification of the components of ethanol extract were performed by GC-MS agilent 6890 N gas chromatography hooked to agilent 5973 N mass selective detector. They equipped with a flame ionization detector and capillary column with HP-5MS (30 m \times 0.25 mm \times 0.25 μ m). In GC settings: the initial oven temperature was set at 60°C for 1 min and ramped at 10° C min⁻¹ to 180° C for 1 min and then ramped at 20°C min⁻¹ to 280 °C for 15 min. The temperature of the injector was controlled at 270°C. The samples (1 μ l) were injected neat, with a split ratio of 1: 10. Helium was used as the carrier gas at a flow rate of 1.0 ml min⁻¹. Spectra were scanned from 20 to 550 *m/z* at 2 scans s⁻¹. Identification of most constituents by gas chromatography was done by comparing their retention indices with those reported in the literature or with those of authentic components available in database.

2.16 Statistical analysis

All values were expressed as mean \pm SD (Standard Deviation). IC₅₀ values were calculated by using GraphPad Prism software. Statistical analysis was performed with one way analysis of variance (ANOVA) followed by Dunnett's 't' test using SPSS statistical software of 20 version. P<0.05 were considered to be statistically significant when compared with control.

3. Results and Discussion

Polyphenolic compounds are the principal antioxidant found in the plant kingdom [30]. Flavonoids, the most common group of phenolic compounds, are responsible for antioxidant, anti-mutagenic and anti-malignant activity [31]. Proanthocyanidins play preventive role in various diseases, like atherosclerosis, gastric ulcer, large bowel cancer, cataracts and diabetes. Flavonols also offer significant cardiovascular health benefits [32-33]. The contents of total phenolics, flavonoids, proanthocyanidins and flavonols of EEACR which were 222.99 \pm 0.41 mg of GAE/g of dry extract, 162.07 \pm 0.79 mg of CAE/g of dry extract, 483.55 \pm 0.75 mg of CAE/g of dry extract and 36.44 \pm 0.25 mg of QUE/g of dry extract, respectively. These results are in good agreement with previous studies on other plant materials [1]. The present study therefore suggests that EEACR may possess diverse pharmacological properties like antioxidant activity due to its

high polyphenolic content.

The total antioxidant capacity (TAC) is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of green phosphate/Mo (V) complex at acid pH [34]. The extract was found to increase the total antioxidant activity with the increasing concentration of the extracts (Table 1). Ferric-ferricyanide complex is reduced to the ferrous form due to the presence of antioxidants in the plant extract [34]. The reducing activity increased with the increasing concentration of the extracts (Table 1).

DPPH assay is generally performed to screen the hydrogen or electron donating capability of a compound or extract. DPPH is a stable free radical which receives hydrogen or electron from an antioxidant compound and gets reduced, resulting in the decolorization of the solution from deep purple or violet to yellow color. The higher is the degree of discoloration, the greater is the antioxidant potential [35]. Figure 1A shows the free radical scavenging activity of EEACR and standard catechin. At a concentration of 50 μ g/ml, the scavenging activity of EEACR was 80.25%; whereas at the same concentration, the standard catechin was 97.42% (Fig 1A). The IC₅₀ of EEACR and catechin were 6.044 and 2.097 μ g/ml, respectively (Table 2). DPPH activity of EEACR exhibited a strong and positive correlation with its total phenolics (R²=0.997, p<0.05) and flavonoids (R²=0.968, p<0.05) contents suggesting the probable reduction of the DPPH radicals by the hydrogen donating ability of phenolic and flavonoid rich EEACR [1]. EEACR can react with free radicals to generate more stable products, ceasing radical chain reactions.

The ABTS cation radical reacts with a hydrogen donating antioxidant and therefore the solution is decolorized. The ABTS assay has been extensively used to measure the total antioxidant potential of plant extracts [35]. Figure 1B shows the ABTS scavenging activity of EEACR, and catechin (standard). At the concentrations of 25, 50 and 100 μ g/ml, the scavenging activity of the EEACR were 78.00%, 85.74% and 91.85%, respectively, while at the same concentrations, the activity of catechin were 67.24%, 88.10% and 93.27%, respectively. The IC₅₀ of EEACR and catechin were 5.266 and 3.843 μ g/ml, respectively (Table 2). The Pearson's correlation coefficients between ABTS radical scavenging and total flavonoids was 0.992 (p>0.05), and that between ABTS radical scavenging and total phenolics was 0.972 (p>0.05).

Nitric oxide is generated in biological tissues by specific nitric oxide synthases [36]. In buffered saline, sodium nitroprusside reacts with oxygen to give rise to nitrite ions that can be measured by using Griess reagent [37]. At a concentration of 50 μ g/ml the percent inhibition values of EEACR and catechin were 71.42% and 72.89%, respectively (Fig 1C). The IC₅₀ of EEACR and catechin were 25.13 and 3.186 μ g/ml, respectively (Table 2). Nitric oxide activity of this extract was also found to be highly correlated with the content of phenolics (R²=0.903, p<0.05) and flavonoid (R²=0.992, p<0.05).

Iron can play role in the generation of hydroxyl radicals by lipid peroxidation. Foods commonly contain ferrous ions which are considered as prooxidants. When ferrozine form complexes with the ferrous ion, a violet color is appeared. The formation of this color can be interrupted by metal chelating agents. Iron chelating ability may be involved in antioxidant activity and affect other functions that contribute to antioxidant activity. So the chelating ability of plant extract on ferrous ion may influence other scavenging activities of

free radicals which protect the organisms against oxidative damage [38]. At the concentrations of 25, 50 and 100 $\mu\text{g/ml}$, the scavenging activity of the EEACR were 34.87%, 43.27% and 59.06%, respectively, while at the same concentrations, the activity of EDTA were 52.59%, 68.20% and 80.43%, respectively (Fig 1E). The IC_{50} of EEACR and EDTA were 63.33 and 23.15, respectively (Table 2). A high value of Pearson's correlation coefficients ($R^2=0.980$, $p<0.05$) indicates a strong relationship between metal chelating activity and phenolics. Flavonoids also showed a strong correlation ($R^2=0.991$, $p<0.05$) with metal chelating scavenging activity.

Lipid peroxidation is a chain reaction in which ROS induce membrane damage by per oxidizing lipid moiety, specially the polyunsaturated fatty acids. Increased levels of lipid

peroxidation have been reported in cancer [39]. Figure 1D showed that the lipid peroxidation inhibition values of EEACR and standard were 68.31%, 78.51% (100 $\mu\text{g/ml}$), respectively. The IC_{50} of EEACR and Catechin were 21.88 and 7.194 $\mu\text{g/ml}$, respectively. Lipid peroxidation inhibition activity of this extract was also found to be highly correlated with the content of phenolics ($R^2=0.987$, $p<0.05$) and flavonoids ($R^2=0.992$, $p<0.05$).

The chemical profile of this extract that was identified by GC-MS spectrum (fig 2), are summarized in table 3. GC-MS analysis identified an active compound, α -asarone, whose antioxidant activity has been reported by several studies [40]. Active principles including α -asarone and phenolic compound present in the EEACR may contribute to the potent antioxidant activity.

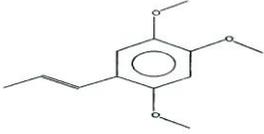
Table 1: Determination of total antioxidant and reducing power capacity of EEACR

Concentration ($\mu\text{g/ml}$)	Absorbance			
	Total antioxidant capacity		Reducing power capacity	
	EEACR	CA	EEACR	CA
3.125	0.098	0.123	0.183	0.217
6.25	0.228	0.246	0.339	0.434
12.5	0.272	0.418	0.591	0.733
25	0.358	0.673	0.819	1.176
50	0.616	1.327	0.912	2.040
100	0.969	1.855	1.345	2.509

Table 2: Antioxidant Activity of EEACR

Name of assay	IC_{50} of EEACR ($\mu\text{g/ml}$)	IC_{50} of Standard ($\mu\text{g/ml}$)
DPPH radical scavenging activity	6.044	2.097 (Catechin)
ABTS radical scavenging activity	5.266	3.843 (Catechin)
Nitric oxide radical scavenging activity	25.13	3.186 (Catechin)
Ferrous chelating assay	63.33	23.15 (EDTA)
Lipid peroxidation inhibition assay	21.88	7.194 (Catechin)

Table 3: Chemical constituent of ethanol extract from *Alpinia calcarata* rhizome

Name of compound	Retention time	Chemical structure
α -asarone	14.4	

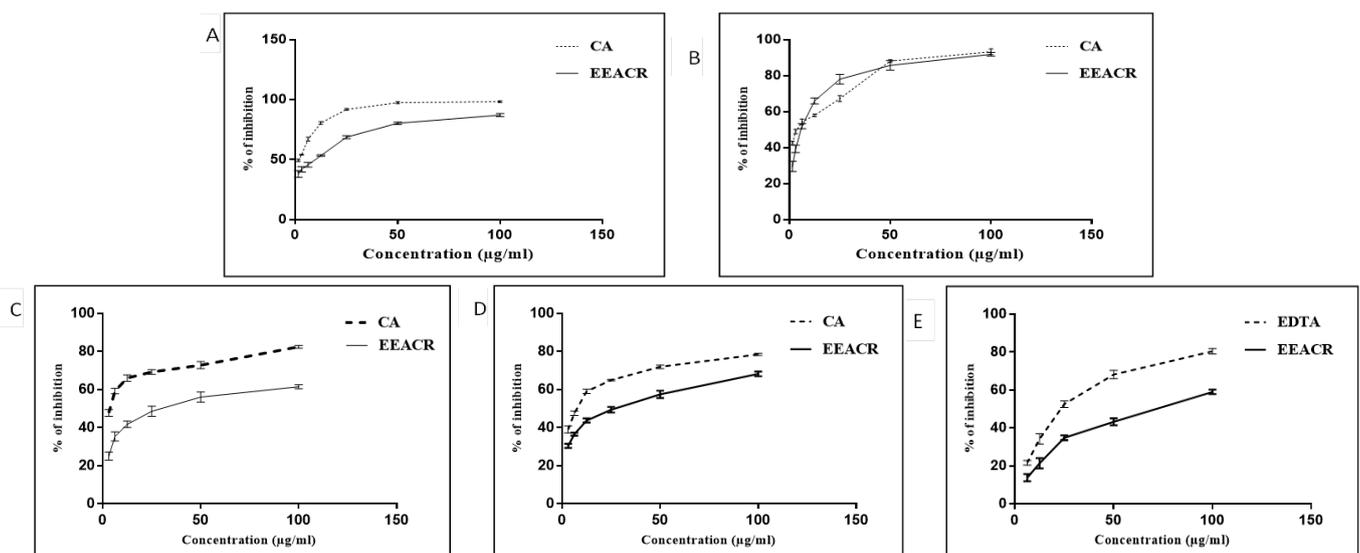


Fig 1: (A) DPPH radical scavenging activity, (B) ABTS radical scavenging activity, (C) Scavenging activity of nitric oxide, (D) Lipid peroxidation inhibition assay, (E) Metal chelating assay of EEACR. Data expressed as mean \pm SD (n=3).

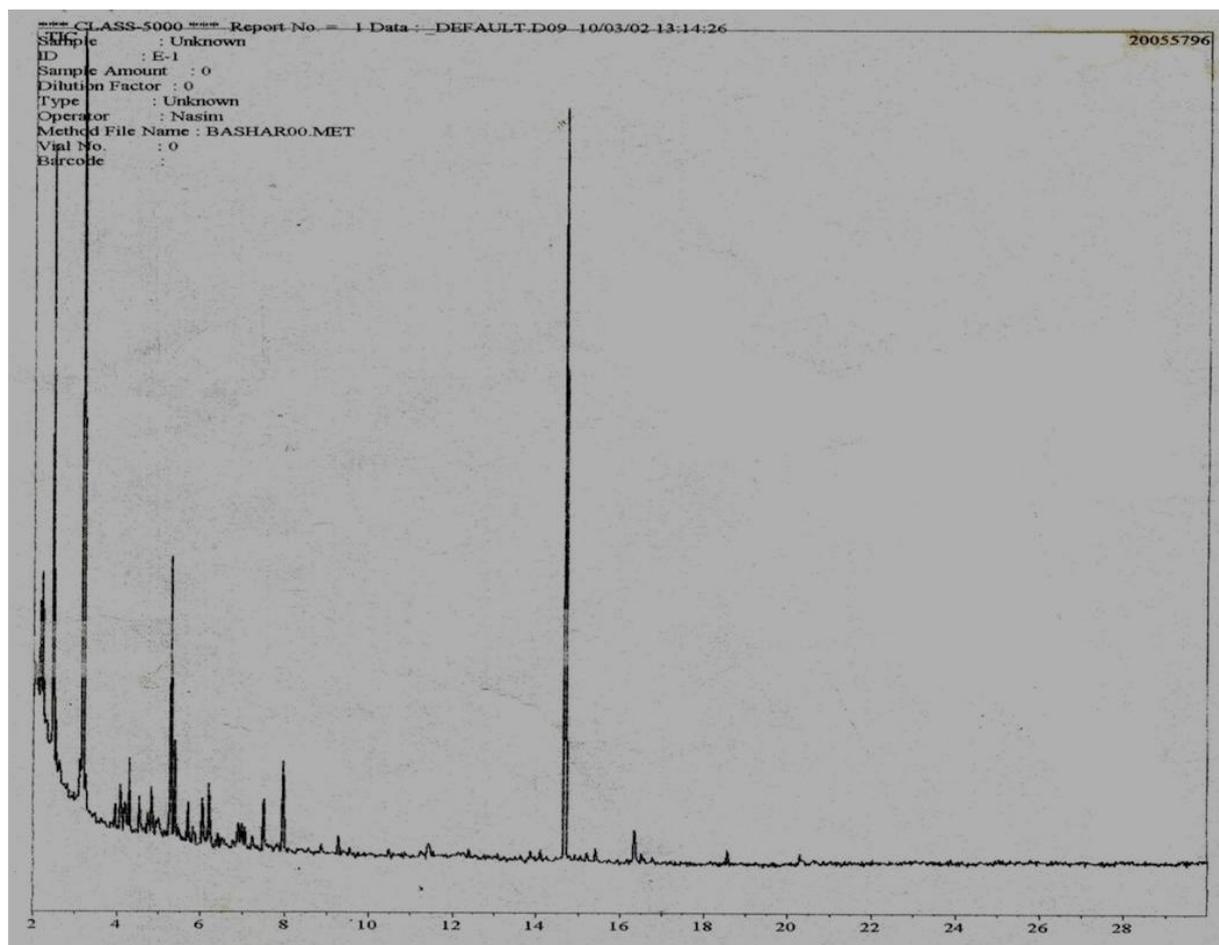


Fig 2: GC-MS chromatogram of ethanol extract of *Alpinia calcarata* rhizome.

4. Conclusion

The present study concludes that ethanol extract of *Alpinia calcarata* rhizome possesses strong antioxidant activity which is comparable to the commercial antioxidants agents and may be helpful for the treatment of various human diseases. In future, investigation is required to identify and characterize all other antioxidant active compounds present in the extract.

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