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Full Length Research Paper

*Syzygium cumini (*pomposia*)* active principles exhibit potent anticancer and antioxidant activities

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The antioxidant and anticancer activities of *Syzygium cumini* fruit extracts were investigated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging assay and viability of leukemia cancer cells (AML cell line) respectively. The successive extracts, hexane, chloroform, ether, ethyl acetate, ethanol and water were prepared and subjected to antioxidant and anticancer evaluation. The results showed that the ethanol extract had stronger antioxidant and anti- leukemia activities than the other ones. Spectroscopic methods data of active ingredients separated from ethanol extract indicated that *S. cumini* fruit extracts contained phenolic compounds, such as Kaempferol 7-O-methylether and sterols such as γ-Sitosterol responsible for their antioxidant and anticancer activities. A significant linear relationship between anticancer potency, free radical-scavenging ability and the content of active compounds of fruit extracts supported this observation.

Key words: Anticancer, antioxidant, successive extraction, *Syzygium cumini*.

INTRODUCTION

“Phyto” is the Greek word for plant. There are many “families” of phytochemicals and they help the human body in a variety of ways. Phytochemicals may protect human from a host of diseases. They are non-nutritive plant chemicals that have protective or disease preventive properties. Plant produces these chemicals to protect itself but recent research demonstrates that many phytochemicals can protect humans against diseases. There are many phytochemicals in fruits and herbs and each works differently (Kumar et al., 2009).

Consumption of fruits and vegetables is shown to lower the risk for chronic diseases such as cancer, cardiovascular diseases and stroke (Yeum et al., 2003). Recently, phytochemicals and their effects on human health have been intensively studied. In particular, a search for antioxidants, hypoglycemic agents, and anticancer agents in vegetables, fruits, teas, spices and medicinal herbs has attracted great attention.

Syzygium cumini (L.) skeels has been attributed in the Indian folklore medicine system to possess several medicinal properties (Warrier et al., 1996). The bark of the plant is astringent, sweet, refrigerant, carminative, diuretic, digestive, antihelminthic, febrifuge, constipating, stomachic and antibacterial. The fruits and seeds are used to treat diabetes, pharyngitis, spleenopathy, urethrorrhea and ringworm infection. The leaves have been extensively used to treat diabetes, constipation (Bhandary et al., 1995), leucorrhoea, stomachalgia, fever, gastropathy, strangury and dermopathy (Warrier et al., 1996), and to inhibit blood discharges in the faeces (Bhandary et al., 1995). The plant possesses acetyl oleanolic acid, triterpenoids, ellagic acid, isoquercitin, quercetin, kaempferol and myricetin in different concentrations (Rastogi and Mehrotra, 1990). Most of these compounds have been reported to possess antioxidant and free radical scavenging activities (Tanaka et al., 1998). The chemical composition and antioxidant activity of *S. cumini* fruits have been studied recently Banerjee and Dasgupta, 2005; Benherlal and Arumughan, 2007), but there is scant information about

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(the antioxidant activity of *S. cumini*.

S. cumini (L.) skeels (Synonym: *S. jambolanum*, *E. jambolana*) (Myrtaceae), popularly known in Brazil as “jambolão” (jambolan or java plum in English), is a native tree of the tropics, originally from India and South East Asia. It is widespread in some states of North, Northeast and Southeast Brazil (Grover et al., 2001; Migliato et al., 2006; Migliato et al., 2007 Reynertson et al., 2008) and is used as a popular treatment against various diseases. In Brazil, the bark, fruits, seeds and leaves of this plant are used for the treatment of diabetes and administered in various pharmaceutical preparations (e.g. aqueous or alcoholic extract, decoctions or crude plant juice) (Braga et al., 2007). *S. cumini* seeds have already shown hypoglycemic and antioxidant activities. A decoction of the bark is also used for dysentery and diarrhea. Moreover, *S. cumini* has been shown to have sedative and anticonvulsant effects and a potent central nervous system depressant effect (Pepato et al., 2004).

Therefore, the aim of the current investigation was to evaluate the efficiency of *S. cumini* (L.) (pomposia) extracts and active ingredients as natural antioxidant and anticancer activity.

MATERIALS AND METHODS

Chemicals and plant materials

Butylated hydroxyanisole (BHA), DPPH (2,2-diphenyl-1-picrylhydrazyl) were purchased from Sigma- Aldrich (St. Louis, MO) and other chemicals were of analytical reagent (AR) purity grade.

Source of pomposia

Mature fruits of *S. cumini* (pomposia) were collected at the campus of Faculty of Agriculture, Cairo University Giza, Egypt (Season July 2009) and identified by Dr. Narmein Shanan, Ornamental Department, Faculty of Agriculture, Cairo University. The ripened fruits were freshly harvested and used for extraction.

Extraction procedure

100 g of fruits were subjected to extraction with successive selective solvents (Rossenthaler, 1930). Hexane, chloroform, diethyl ether, ethyl acetate, ethanol and distilled water were used. The polarity was increased from non-polar to highly polar. Each solvent extract was evaporated to dryness using rotary evaporator and weighed.

Antioxidant activity

The antioxidant activity of the plant extracts was evaluated by using the 2.2'diphenylpicrylhydrazyl (DPPH) assay (Cuendet et al., 1997; Burits and Bucar, 2000). The extracts were added to 5 ml of a 0.004% (w/v) of DPPH in methanol (100% v/v). After, a 30 min incubation period at room temperature the absorbance at 517 nm was compared to DPPH in methanol without an extract sample (blank). BHA was used as positive control and extracts concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentages against extract concentration.

The percent inhibition of free radical formation (I%) was calculated as

$$I\% = (A_{blank} - A_{sample} / A_{blank}) \times 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the extract) and A_{sample} is the absorbance of the mixture containing the extract. The experiment was carried out in triplicate.

Viability of tumor cells

The study was performed on cells harvested from adult leukemia patients or healthy relatives admitted to the National Cancer Institute, Cairo University. International protocols governing the ethical treatment of patient were followed. The experimental samples were taken from healthy volunteer relatives (3 samples) as normal control and leukemia patients that included AML (acute myeloid leukemia, immature monocytes) patients. Mononuclear cells were separated from other blood cells by Ficoll hypaque density gradient (Pharmacia, Uppsala, Sweden). The cells were then washed with three changes of PBS. The cell counts were adjusted so 10^5 cells in 0.1 ml (counting both mature and immature cells). The culture medium was prepared using modified Earle’s salt with 1.2 g/l sodium carbonate and L-glutamine (Gibco, Grand, USA), 10% (v/v) inactivated fetal bovine serum (Gibco), 100 µg /ml penicillin and 100 µg/ml streptomycin were added. The medium was filtered through 0.22 µm millipore filter, one ml of which was transferred into a 1.8 ml screw-capped sterile plastic tube. Next, 0.1 ml of the cell suspension containing 10^5 cells was added to each of tube per extract. To one of the tubes, 0.1 ml of the extract was added, while the other two tubes served as negative and positive controls respectively. Culture medium was used instead of the extract for the negative control and the extract was added to the cells from healthy volunteers as a positive control. The tubes were incubated at 37°C in the presence of 5% (v/v) CO_2 for 24 h (dark condition, humidified air). The cells were tested for their viability using the trypan blue exclusion test (Bennett et al., 1976). Two hundred cells were counted, and the percentage of viable cells was estimated. The extracts concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentages against extract concentration.

Separation of active gradient

10 g of *S. cumini* crude ethanolic extract were fractionated over a Vacuum Liquid Chromatographic Column (VLC, 15 x10 cm and packed with VLC silica gel H (100g). Gradient elution was carried out with hexane, chloroform and their mixture with an increased polarity pattern (100% hexane, chlorform to 100% ethyl acetate and finally with 100% acetone). Fractions (200 ml of each) were collected (Figure 1). Each was separated, evaporated under reduced pressure to dryness, redissolved in 5 ml of ethanol and monitored by TLC, using the solvent system toluene, ethyl acetate (97: 3, v/v). The TLC chromatogram was visualized under ultra violet light at 365 nm and 245 nm before exposure to anizaldehyde reagent.

The most potent fractions were chosen for further identification using the chromatographic and spectroscopic methods as follows:

a-MS Analysis of potent fractions

The potent fractions of *S. cumini* were analyzed by mass spectrum (MS). The mass spectrometer was scanned over the 40 to 500 m/z range with an ionizing voltage of 70 eV and identification was based on standard mass library of National Institute of Standards and

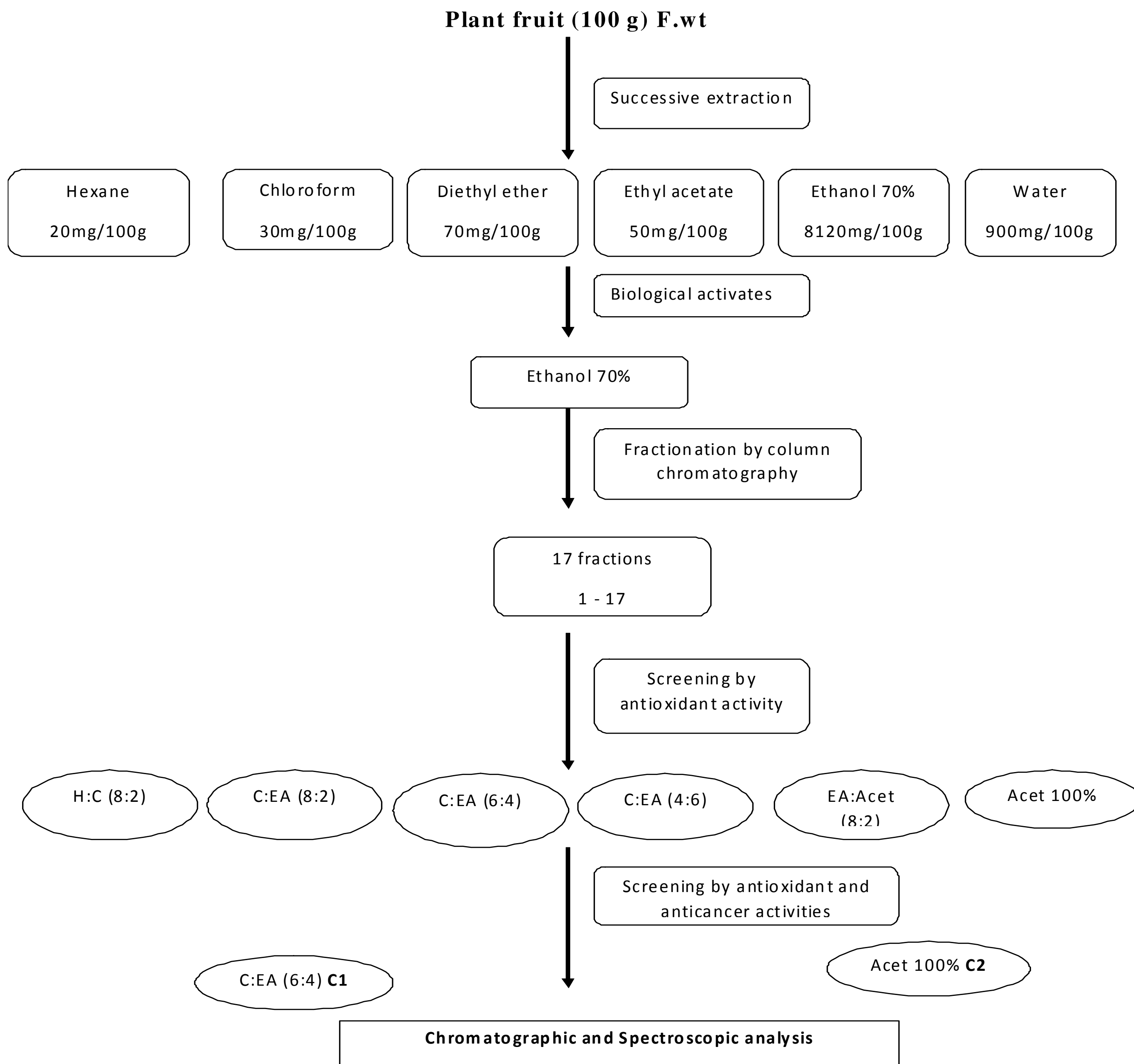

Figure 1. Scheme for fractionation of ethanol fraction of *Syzygium cumini*.

Technology (NIST Version 2.0) to detect the possible fraction structure.

b-FTIR spectra

A Perkin Elmer (Waltham, Massachusetts, USA) was used to obtain Fourier transformed infrared (FTI**R**) spectra (System 2000).

Statistical analysis

Statistical analysis (standard deviation ''SD'' and standard error (''SE'') was carried out according to Fisher (1970). LSD (Least significant difference) test was used to compare the significant differences between means of treatment Waller and Duncan (1969). The statistical package for social science S.P.S.S. (1999) program version was used for all analysis.

RESULTS AND DISCUSSION

Antioxidant activity

Many aromatic plants and spices especially clove buds (*S. cumini)* and their active ingredients have been known

Table 1. Antioxidant activity of successive extract from *Syzygium cumini.*

Solvent	Scavenging activity (%)		
	25 µg/ml	50 µg/ml	100 µg/ml
Hexane	13.36±1.0	23.33±2.3	34.41±1.7
Chloroform	8.23±0.5	19.38±1.5	50.65±2.6
Ether	16.565±0.7	26.125±1.7	71.07±3.3
Ethyl acetate	49.99±2.6	75.155±4.1	81.07±2.7
Ethanol	50.23±3.2	74.66±3.0	81.805±1.4
Water	9.88±0.1	29.245±2.5	30.86±1.2
BHA	49.67±2.1	77.1±1.9	85.6±2.1
LSD 0.05	4.31	5.62	3.85

The values are means ± SE. Each value is presented as mean of triplicate treatments, LSD: Least different significantly at $p \leq 0.05$ according to Duncan's multiple range test.

Table 2. Anticancer activity of successive extract from *Syzygium cumini*.

Solvent	Anticancer activity (%)		
	25 µg/ml	50 µg/ml	100 µg/ml
Hexane	13.17±0.3	19.0±2.0	32.2±2.1
Chloroform	18.77±1.1	25.7±1.8	43.0±1.6
Ether	12.03±1.0	16.1±0.5	25.6±0.6
Ethyl acetate	16.47±1.5	23.2±1.1	39.9±2.6
Ethanol	21.53±2.0	36.5±1.9	70.7±2.4
Water	16.87±1.7	32.8±2.2	51.5±3.5
LSD 0.05	1.94	3.07	5.92

The values are means ± SE. Each value is presented as mean of triplicate treatments, LSD: Least different significantly at $p \leq 0.05$ according to Duncan's multiple range test.

to support various biological activities such as antimicrobial and antioxidant properties (Fu et al., 2007). The radical scavenging effects (percentage of quenched radicals) were determined for clove buds extracts and their constituents. The clove buds extracts or their constituents when mixed with DPPH decolorized it due to hydrogen donating ability. All the tested samples (*n*-hexane, chloroform, ether, ethyl acetate, ethanol and water extracts as well column fractions and pure compounds) revealed scavenging effects on DPPH (13-70%) (Table 1). Antioxidants are believed to neutralize the free radicals in lipid chains by contributing a hydrogen atom usually from a phenolic hydroxyl group, which in turn converts phenolic groups into stable free radicals that do not initiate or propagate further oxidation of lipids (Shalaby et al., 2010). It was observed that the scavenging activity of extract of clove buds at all concentrations from 25 to 100 µg/ml is rather strong (13 - 70%). The antioxidant activities of fractions separated from ethanolic extract by column chromatography have various antioxidant activities against DPPH. The results showed that, fractions 2, 6, 8, 9, 11 and 17 had the strongest anti DPPH radical activity (30.2-62.0 %) (Table 3). These fractions were used for different biological activities (antioxidant and anticancer activity). The promising fractions (8 and 17) were used for the separation of the active compounds. The identification of their chemical structure proved as γ-sitosterol and Kaempferol 7-O-methylether respectively. The remarkable antioxidant activity of pure compounds might be due to the double bond and hydroxyl group (Figure 2). These results are in accordance with literature work of Lee and Shibamoto (2001). The isolated flavonoids of *S. cumini* buds (compound no. 17, Kaempferol 7-O-methylether; IC_{50} =85.4 µg/ml) were found to act as strong free radical scavengers (Table 4) and these results are in agreement with the results obtained by Teffo et al. (2009) who reported that four kaempferol methyl ethers demonstrated varying degrees of antioxidant activity against DPPH radical. But γ-sitosterol (compound no. 8, IC_{50} = 88.5 µg/ml) demonstrated quite strong antioxidant activity against DPPH radical as reported by Weng and

Table 3. Antioxidant activity of successive extract from *Syzygium cumini.*

Fraction numbers	Fractions	Scavenging activity (%)	
		50 µg/ml	100 µg/ml
1	Hexane100%	20.1±1.0	34.3±2.1
2	Hexane:Chloroform 8:2	25.0±0.5	39.55±0.3
3	Hexane:Chloroform 6: 4	2.3±0.0	4.4±0.1
4	Hexane:Chloroform 4: 6	16.7±0.3	26.85±2.1
5	Hexane:Chloroform 2: 8	3.3±0.6	3.6±0.0
6	Chloroform 100%	17.1±1.0	30.2±1.5
7	Chloroform: Ethyl acetate 8:2	8.8±0.0	17.55±0.9
8	Chloroform: Ethyl acetate 6: 4	31.5±1.2	62±2.2
9	Chloroform: Ethyl acetate 4: 6	19.9±0.4	37.95±2.8
10	Chloroform: Ethyl acetate 2:8	10.2±0.8	15.6±1.0
11	Ethyl acetate 100%	26.6±1.5	33.4±2.1
12	EA: Acetone 8:2	14.7±0.4	27.6±0.3
13	EA: Acetone 6: 4	3.3±0.0	8.15±0.1
14	EA: Acetone	11.8±0.5	25±1.2
15	EA: Acetone 2: 8	2.5±0.1	6.7±0.4
16	EA: Acetone 1:9	3.0±0.2	6.9±0.0
17	Acetone 100%	41.4±1.7	59.3±1.4
LSD 0.05		3.84	3.37

The values are means ± SE. Each value is presented as mean of triplicate treatments, LSD: Least different significantly at $p \leq 0.05$ according to Duncan's multiple range test.

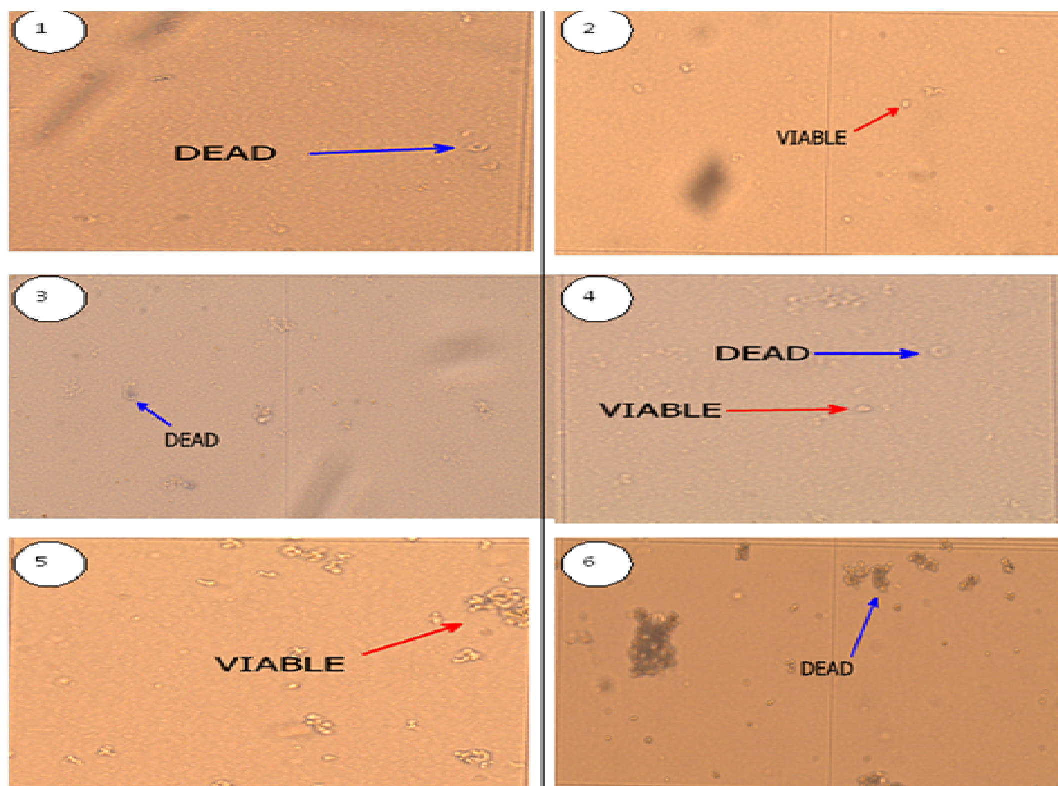

Figure 2. Effect of different pure compounds isolated from *Syzygium cumini* on leukemia cancer cell (AML cell line).

Table 4. Antioxidant and anticancer activity of pure compound separated from the promising fractions for ethanolic extract of *Syzygium cumini* (100 µg/ml).

Compound number	Activity (%)	
	Antioxidant activity	Anticancer activity
1 (Fr. 2)	37.2±2.1	89.6±3.2
2(Fr. 6)	33.8±1.8	9.7±0.4
3(Fr. 8)	53.4±2.7	91.71±2.9
4(Fr. 9)	29.35±1.1	84.5±2.6
5(Fr. 11)	23.05±0.8	0±0.0
6 (Fr. 17)	53.7±1.3	100±0.5
LSD 0.05	6.96	17.36

The values are means ± SE. Each value is presented as mean of triplicate treatments, LSD: Least different significantly at $p \leq 0.05$ according to Duncan's multiple range test.

Wang (2000).

Anticancer activity

The six successive extracts and six purified active principles from *S. cumini* fruit extracts were tested for their antileukemic activity on AML cells. The results summarized clearly indicates that tested extracts and active principles exhibited a significant dose-dependent inhibitory effect on cancer cell lines examined (Tables 2 and 4). Results indicated that in human AML the ethanolic extract have the highest anticancer activity (LC_{50} = 81 µg/ml) when compared with other extracts (70.7 %) but less than the pure compounds (γ-sitosterol (LC_{50})= 55.0 µg/ml) and Kaempferol 7-O-methylether ((LC_{50})= 48.0 µg/ml) which have 91.71 and 100 % inhibition respectively. These results may be due to antagonism effect between the different active ingredients present in ethanol crude extract.

These results are in agreement with the results obtained by Rastogi and Mehrotra (1990) who found that Kaempferol compound posses antioxidant and anticancer activity. Kaempferol inhibits cell proliferation by disrupting the cell cycle, which is strongly associated with the induction of arrest at G2/M phase and may induce apoptosis via p53 phosphorylation in human breast carcinoma MDA-MB-453 cells (Choi and Ahn, 2008). In addition, Lucena et al. (2009) who found that antitumor activity of *Cissus specie* can be related to the presence of sitosterol which is pointed out as inducing apoptosis together with TNF-α and antitumor activity.

Pure compounds

γ-sitosterol

The IR spectrum of C1 compound showed absorption at 3300 (OH), region between 2937-2865 ($-CH_2$ and CH_3 group), 2349, 662 and 630 cm^{-1}. Cholesterolic OH present also on intense band at 1049 cm^{-1} that corresponds IR to the secondary OH. As shown in mass spectrum, molecular ion of m/e= 412 is consistent with the molecular formula of $C_{29}H_{50}O$, m/e of 354 $(M-H_2O-C_3H_7)^+$, 294 $(M-H_2O-C_7H_{15})^+$, 245 (M-side chain $(C_{10}H_{21}))^+$, 206 ($M-H_2O$-side chain $(C_{13}H_{25}))^+$, 168 $(C_{12}H_{24})^+$, 138 ($C_{12}H_{18}$), 107 (C_8H_{11}), 79 (C_6H_7). The structure of C1 was elucidating as γ-sitosterol (Figure 3 and Figure 4). Also, conformation of this structure was confirmed, by comparison of their mass spectra with those of previously reported in literature by Goad and Akihisa (1997).

Kaempferol 7-O-methylether

The IR spectrum of C2 compound showed absorption at 3260 (OH), 1663 (unsaturated C=O), 1609, 1587 and 1504 cm^{-1} (C=C). As shown in mass spectrum, molecular ion of m/e= 300 is consistent with the molecular formula of $C_{16}H_{12}O_6$, and the following fragments appear after fragmentation of compound; 285, 271, 257, 229, 167, 121 and 105. The structure of C2 was elucidating as Kaempferol 7-O-methylether (Figures 3 and 5). Also, compounds structures were confirmed, by comparison of their mass spectra with those of previously reported in literature by Son et al. (1998).

CONCLUSION

The ethanol extract from *S. cumini* fruits have highest antioxidant and anticancer activity and this activity was correlated with the presence of different active ingredients especially γ-sitosterol and Kaempferol 7-O-methylether.

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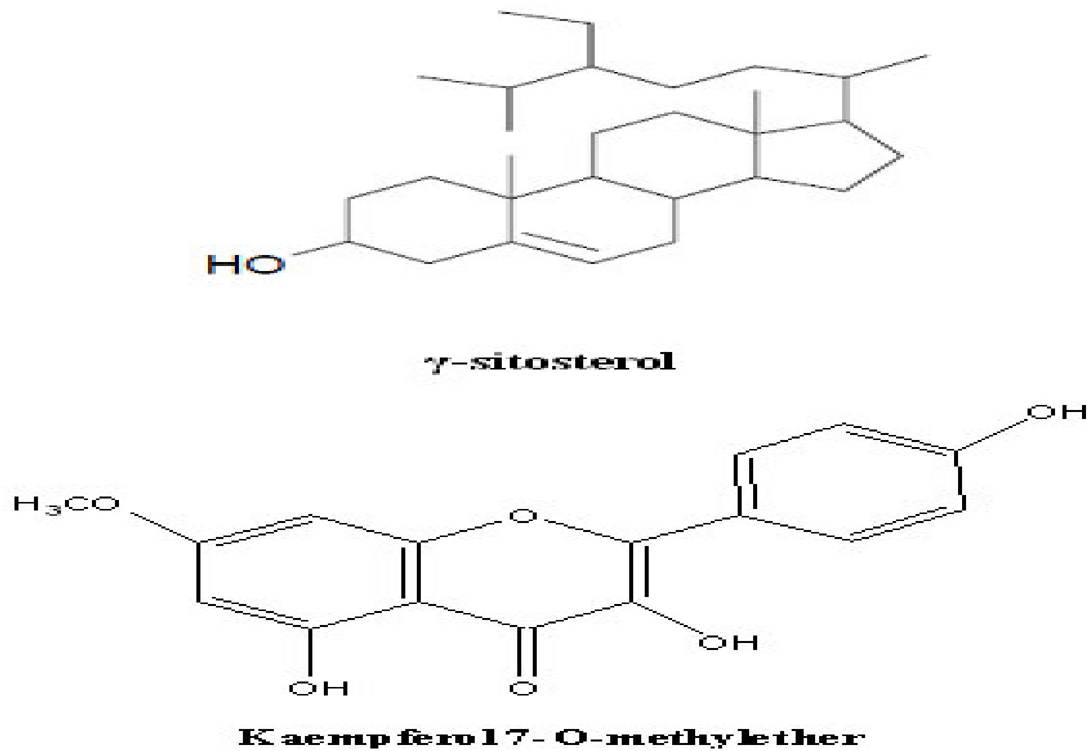

Figure 3. Suggested chemical structure of active ingredients which separated from ethanolic extract of *Syzygium cumini.*

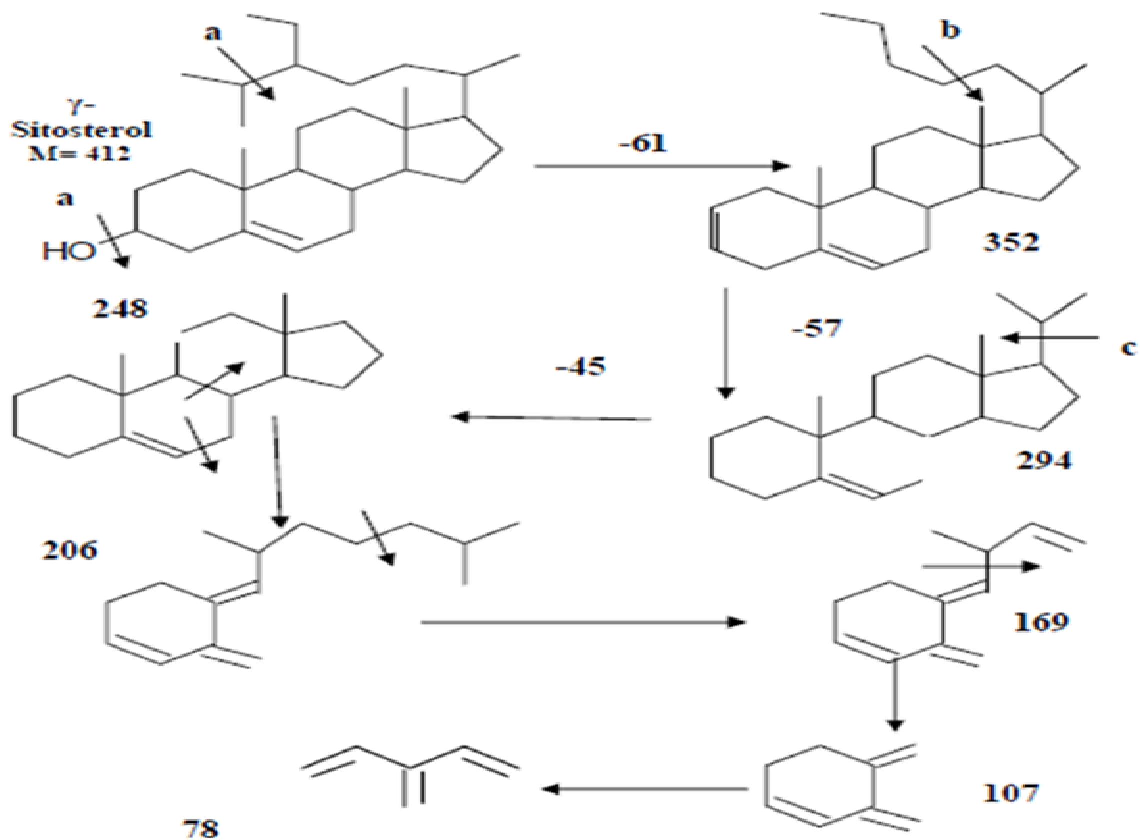

Figure 4. The fragmentation pattern of γ-sitosterol.

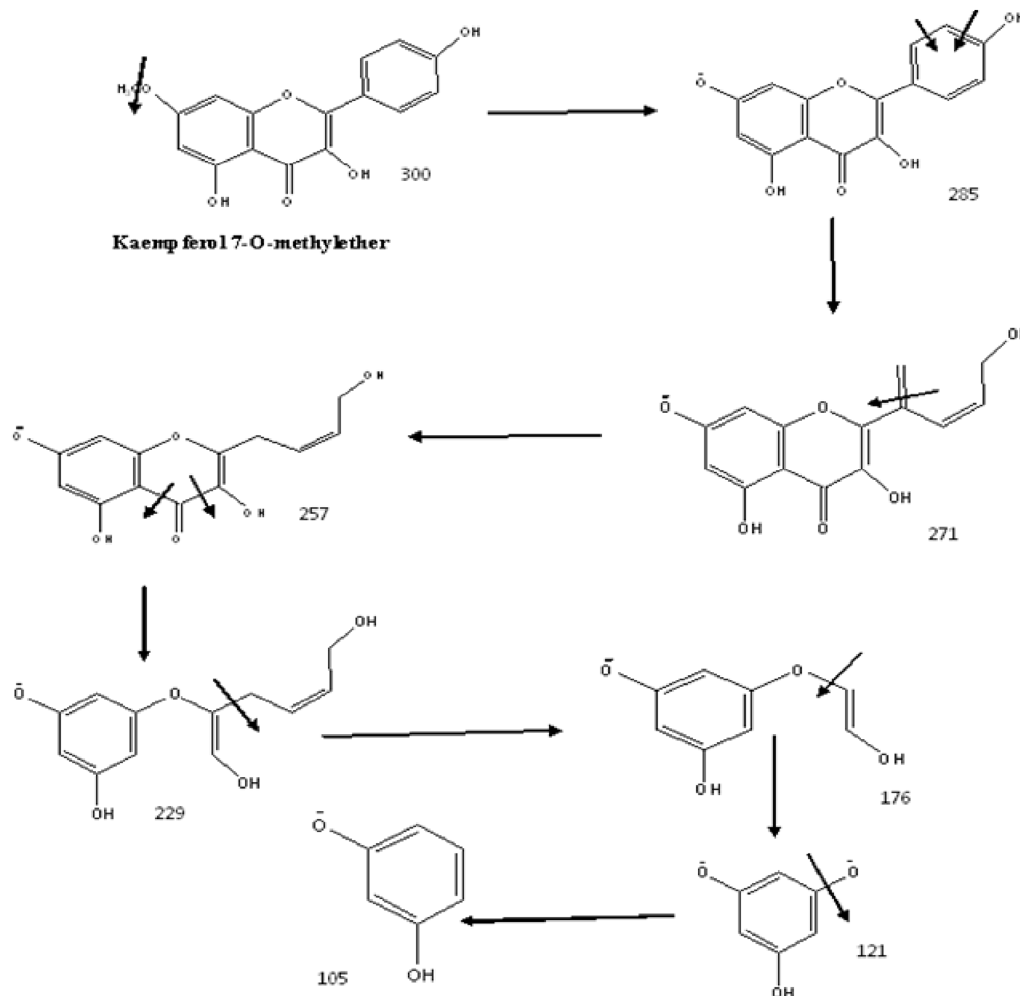

Figure 5. The fragmentation pattern of Kaempferol 7-O-methylether.

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