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Volatile constituents and *in vitro* activity of *Syzygium aromaticum* flower buds (clove) against human cancer cell lines

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Abstract: The methanolic extract (SA-EXT) of *Syzygium aromaticum* flower buds and its fractions tested against three human cancer cell lines *viz* uterine cervix (HeLa), breast (MCF-7) and lung NCI (H-460) using sulforhodamine-B assay. The ethyl acetate soluble sub fraction (SA-EAS) was active only against HeLa cells with GI_{50}value of 36± 3.4μg/mL. The most active sub-fraction (SA-PES-Fr-2) showed growth inhibition (GI_{50}: 36, 50 and 68µg/ml against MCF-7, HeLa and NCI-H-460 cancer cell lines, respectively) with cytotoxic effect LC_{50}= 88 ± 3.4 μg/mL against HeLa and LC_{50}=86 ± 2.8 μg/mL against MCF-7. The most active sub-fraction (SA-PES-Fr-2) analyzed by GC and GC-MS techniques revealed that eugenol was most abundant (85.34%) along with minor constituents. Thus it can be concluded that growth inhibitory and cytotoxic effect residing in *Syzygium aromaticum* flower buds (clove) is more likely due to eugenol.

Keywords: Cytotoxicity, growth inhibition, *Syzygium aromaticum*, eugenol, GC-MS, sulforhodamine-B.

INTRODUCTION

Cancer is the second leading cause of death for men and women worldwide, with approximately 14 million new cases in 2012 (Torre *et al*., 2015) and 8.8 million deaths occurred in 2015 (Ferlay *et al*., 2015). Global incidence of cancer is rising per year (Jemal *et al*., 2005). Infections with certain viruses, bacteria and parasites are major risk factor for specific cancers. Carcenogenic infections in humans are an important cause of cancer, particularly in less developed countries, reported by the International agency for research on cancer (IARC) including *Helicobacter pylori* ,hepatitis B virus (HBV), hepatitis C virus (HCV), HIV-type 1, human papilloma virus etc (Plummer *et al.*, 2016).

Lung cancer is the leading cause of cancer mortality in most countries (Sethi, 2002) and tobacco smoking has been identified as the major risk factor (Hecht *et al*., 1993). Benzo [*a*] pyrene (BP), one of the important tobacco related carcinogen involved in the formation of DNA adducts that ultimately leads cancer (King *et al.*, 1979). Breast cancer most commonly occurring cancer in women, comprising almost one third of all malignancies (Kumar *et al*., 2014) while, cervical cancer is the fourth most common cancer in women worldwide. There were an estimated 266,000 deaths from cervical cancer worldwide in 2012, accounting for 7.5% of all female cancer deaths. Almost nine out of ten (87%) cervical cancer deaths occur in the less developed regions (Torre *et al*., 2015).

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Although, several compounds are currently employed in cancer treatment, but due to various side effects and development of resistance has limit their uses and an ideal anticancer drug has not been discovered as yet. Therefore, search for new efficacious and safe anticancer compounds with the understanding of their mechanism of action, many research groups are focusing on alternative sources including medicinal plants.

Natural products have played an important role as an effective source of antitumor agents. About 30-40% of the anticancer drugs used globally are derived from plant sources (Newma *et al*., 2003). The investigation of medicinal plants continues to find additional anticancer drugs with minimum or no side effects (Huang *et al*., 2010).

Clove, the dried flower buds of *Syzygium aromaticum* (family Myrtaceae) has been widely used as a spice as well as in traditional Chinese and Indian medicine systems. It is also used for dyspepsia, vomiting, nausea and gastric ailments. Clove oil is a powerful antiseptic, local anesthetic and analgesic, expectorant, antioxidant, stomachic and spasmolytic (Khare, 2008). It is also used in few anticancer formulations (Kenner and Requena, 1996). It has been reported that extracts and oil of clove have significant activity against different cancer cell lines such as ovarian cancer cells (SKOV-3), cervical epithelial cells (HeLa), liver cancer cells (BEL-7402), colon cancer cells (HT-29), breast cancer cells (MCF-7), pancreatic cells (PANC-1), normal colon epithelial cells (CCD 841 CoN), normal lung fibroblasts (IMR-90), human normal dermal fibroblasts (HNDF) and HMEC-1 cells, an SV40

transformed human dermal microvascular endothelial cell lines (Liu *et al.,* 2014; Prashar, Locke, & Evans, 2006). Phytochemistry of *Syzygium aromaticum*is well researched and several compounds such asflavonoids (Nassar, 2006), chromones (Ah-Reum and Young-Sook, 2010), ellagitannins (Tanak *et al.*, 1996) triterpenoids(Brieskorn *et al*., 1975), steroids (Charle *et al.*, 1998), sesquiterpenes, monoterpenes and phenylpropanoids (Carvalho *et al.*, 2015) have been reported. Eugenol is the active component of clove oil having antimutagenic, antigenotoxic, anti-inflammatory and cytotoxic properties against colon cancer (SNU-C5), human promyelocyticleukemia (HL-60) and liver (HepG2) cell lines (Blowman *et al.*, 2018; Pal *et al.*, 2010; Yoo *et al.*, 2005). Ethyl acetate soluble fraction of methanolic extract (SA-EXT) containing a substantial quantity of triterpeneoleanolic acid has been associated with antiproliferative and *in vivo* antitumor activity (Liu *et al.*, 2014).

In the present study the methanolic extract, its fractions and purified sub-fraction of clove were evaluated for growth inhibition and cytotoxic activities against three human cancer cell lines i.e. uterine cervix (HeLa), breast cancer (MCF-7) and lung cancer NCI (H-460). This is the first report on anticancer activity directed fractionation and identification of active constituents of clove. The most active oily petroleum ether soluble sub-fraction (SA-PES-Fr-2) was analyzed by GC-MS.

MATERIALS AND METHODS

Cells, chemicals and reagents

Dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), gentamycin sulphate, $_L$-glutamine penicillin streptomycin solution (GPSS), Roswell Park Memorial Institute-1640 medium (RPMI-1640), sulforhodamine B (SRB), trichloroacetic acid (TCA), tris base, trypan blue and trypsin-EDTA (Sigma Co St. Louis, Mo, USA), acetic acid (Lab scan, Ireland) and doxorubicin (ICN, USA) were purchased from respective manufacturers.

Preparation of clove extracts and fractions

Dried spice (clove; 5kg) (fig. 1) was purchased from local market of Karachi, Pakistan. Itwas identified by Dr. Jan Alam of Department of Botany, University of Karachi and a voucher specimen was deposited in the Herbarium of the same department (KUH-GH No. 01). They were milled to fine powder with the aid of an electric blender and repeatedly extracted with methanol (five times) at room temperature. The concentrated extract (SA-EXT) obtained on removal of the solvent under reduced pressure was partitioned between ethyl acetate and water (SA-MAQ). The residue (SA-EAR) obtained from ethyl acetate layer after washing with water and drying with Na_2SO_4 (anhydrous) and removal of the solvent under reduced pressure was divided into petroleum ether soluble (SA-PES) and petroleum ether insoluble fractions. The petroleum ether insoluble fraction was further divided into ethyl acetate soluble (SA-EAS) and ethyl acetate insoluble fractions and the later was again fractionated into acetone soluble (SA-AS) and insoluble portions, which was soluble in methanol (SA-MS). All the fractions of methanolic extract (SA-MAQ, SA-EAR, SA-PES, SA-EAS, SA-AS and SA-MS) were screened for activity against three cancer cell lines; uterine cervix cancer (HeLa), breast cancer (MCF-7) and lung cancer (NCI-H460) (table 1). The petroleum ether soluble fraction (SA-PES, 10g) was subjected to column chromatography (CC) (petroleum ether-ethyl acetate in increasing order of polarity). Six fractions (SA-PES-Fr-1 to SA-PES-Fr-6) were ultimately obtained on combining the eluates on the basis of TLC. The fraction SA-PES-Fr-2 (petroleum ether-ethyl acetate 9:1 eluates) was an orange oil (7.8g) with a characteristic clove odor (fig. 2) demonstrated significant activity against tested cell lines was subjected to GC-MS (table 2; fig. 3).

Fig. 1: Flower buds (Clove) of *Syzygium aromaticum* Linn.

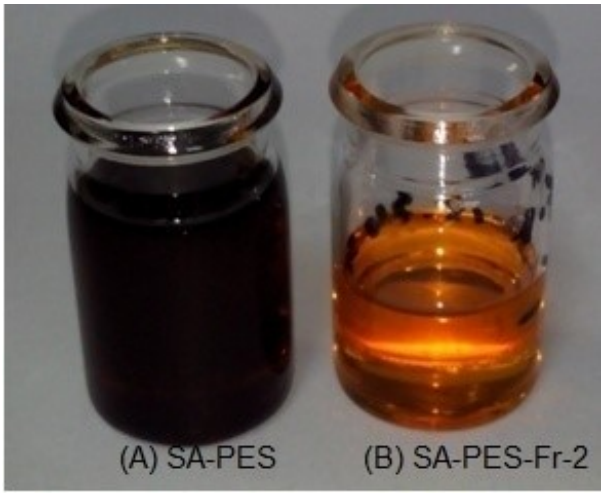

Fig. 2: (A) Petroleum ether soluble fraction (SA-PES) and (B) Petroleum ether soluble sub-fraction (SA-PES-Fr-2).

***Analysis of active sub-fraction* (SA-PES-Fr-2)**

Gas chromatography (GC)

Gas chromatography was carried out using flame ionization detector (FID) on the less polar capillary

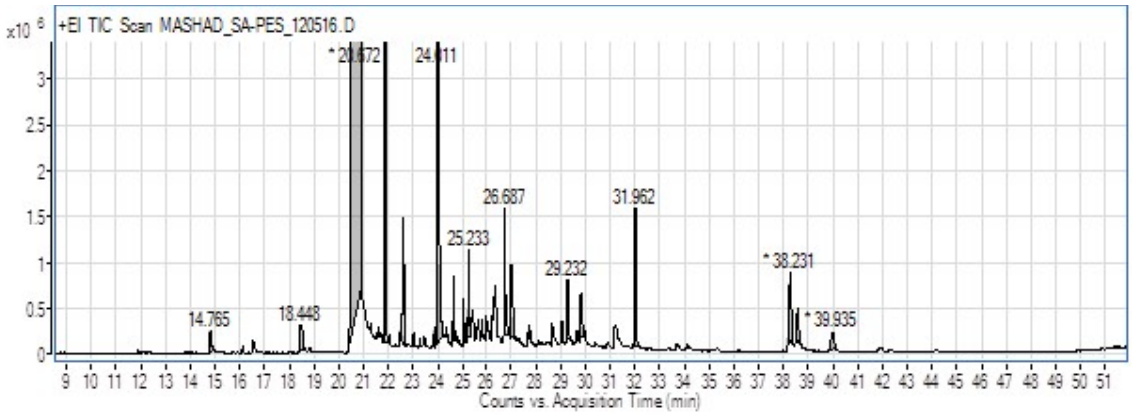

Fig. 3: GC-MS of active petroleum ether soluble fraction (SA-PES-Fr-2)

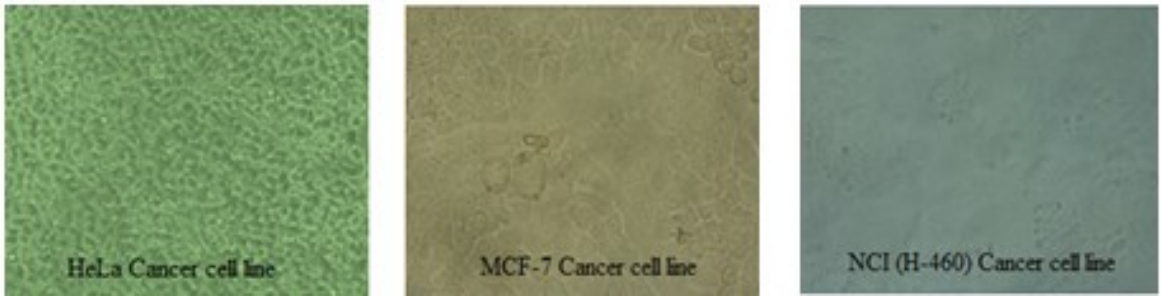

Fig. 4: Light microscope images (10x) of uterine cervix (HeLa), breast (MCF-7) and lung NCI (H-460) cancer cell lines

column Zebron™ ZB-5 (60m x 0.53mm x 0.25µm film thickness of 5%- Phenyl -95% Dimethylpolysiloxane) installed on a Shimadzu 17-AGC system. The analysis was performed with dual temperature program an initial temperature 75°C for 3 minutes then ramped at a rate of 9^0C/ minutes to a final temperature one 200°C with holding time of 5 minutes and again ramped at a rate of 7^0C/ minutes to a final temperature two 240^0C with holding time of 30 minutes Injector with a splitting ratio of 1:10 was set at 240^0C and FID at 260^0C. Carrier and make up gas, nitrogen was maintained at a flow of 10.939 mL/minutes with pressure of 89 kPa.

Gas chromatography-mass spectrometry (GC-MS)

For GC-MS experiments Agilent Technologies 7000 GC/MS Triple Quad gas chromatograph, equipped with ZB-5MS (30m x 0.32 ID and 0.25µm film thickness) was combined with a Jeol, JMS-600H mass spectrometer operating in EI mode with ion source at 250°C and electron energy of 70eV. Carrier gas volume was adjusted between 1.0-5.0mL/min depending upon the detector response. GC-MS analysis was performed at HEJ Research Insstitute of Chemistry International Center for Chemical and Biological Sciences (ICCBS), University of Karachi.

Determination of the constituents

The active fraction (SA-PES-Fr-2; *loc. cit*) was analyzed by gas chromatography using flame ionization detector (GC-FID) and GC-EIMS analysis (Masada, 1976). The components were identified by mass spectral survey (WebBook) and comparison of the mass spectrum of each component obtained by GC-MS, with those reported for the selected compounds.

Determination of growth inhibition and cytotoxicity

The cell growth inhibition and cytotoxicity induced by clove extract and its fractions were evaluated using sulforhodamine-B assay against three human cancer cell lines (fig. 4) uterine cervix (HeLa), breast (MCF-7) and lung (NCI-H460) (Bano et al., 2017; Qamar et al., 2010; Skehan et al., 1990).

Preparation of stock solutions

The stock solutions of clove extract and fractions (40 mg/mL) were prepared in sterile DMSO (100%). The stock solutions for doxorubicin (1mM) was constituted in sterile distilled water and kept at -80ºC until further use. On the day of experiment, all the dilutions were prepared in RPMI-1640 containing gentamicin (50μg/mL).

Sulforhodamine-B assay

It was performed as described earlier (Anita *et al.*, 2012). All three cell lines were trypsinized and seeded in 96-well plate at a density of 10,000 cells/well/100μL and incubated in CO_2 incubator at 37ºC to allow monolayer formation (24h) followed by the addition of various concentrations of methanol extract (SA-EXT) of *S.*

Table 1: Growth inhibition and cytotoxicity effects of methanolic extract of *S. aromaticum* and its fractions against uterine cervix (HeLa), breast (MCF-7) and human lung (NCI-H460) cancer cell lines.

Test agents	Human cancer cell lines (µg/mL)					
	HeLa		MCF-7		NCI-H460	
	GI_{50}	LC_{50}	GI_{50}	LC_{50}	GI_{50}	LC_{50}
Extract						
SA-EXT	136 ± 3.1[c]	>250	180 ± 3.7[c]	>250	110 ± 2.9[c]	>250
Fractions						
SA-EAR	30± 1.1[b]	>100	50± 3.4[b]	90 ± 3.7[b]	76± 2.4[b]	>100
SA-PES	>100	>100	>100	>100	>100	>100
SA-EAS	36± 3.4[b]	>100	>100	>100	>100	>100
SA-AS	>100	>100	>100	>100	>100	>100
SA-MS	>100	>100	>100	>100	>100	>100
SA-MAQ	>100	>100	>100	>100	>100	>100
SA-PES-Fr-2	50 ± 3.1[b]	88 ± 3.4[b]	36 ± 3.1[b]	86 ± 2.8[b]	68 ± 3.1[b]	>100
Reference drug						
Doxorubicin µg/mL (µM)	0.5 ± 0.02[a] (0.88 ± 0.04)	5.8 ± 0.1[a] (10 ± 0.1)	0.17 ±0.03[a] (0.3 ± 0.05)	5.8 ± 0.01[a] (10 ± 0.02)	0.17 ± 0.05[a] (0.26 ± 0.08)	5.4 ± 1.2[a] (9.3 ± 1.2)

Methanolic extract of clove (SA-EXT), Main ethyl acetate residue (SA-EAR), Petroleum ether soluble fraction (SA-PES), Ethyl acetate soluble fraction (SA-EAS), Acetone soluble fraction (SA-AS), Methanol soluble fraction (SA-MS), Main aqueous fraction (SA-MAQ), Petroleum ether soluble sub-fraction (SA-PES-Fr-2).
Control absorbance (515 nm): HeLa (2.3 ± 0.5), MCF-7 (2.9 ± 0.5) and NCI-H460 (2.9 ± 0.5)
Each value represents mean ± SEM of three independent experiments
Concentration causing 50 % of cell growth inhibition = GI
Concentration of drug that killed 50% cells = LC_{50}
Values within parentheses are expressed in µM.
In columns, dissimilar superscript alphabets (a-c) represent significant (p<0.05) and similar alphabets non-significant IC_{50} and LC_{50} values.

aromaticum (10 - 250μg/mL) and its fractions SA-EAR, SA-MAQ, SA-PES, SA-EAS, SA-AS and SA-MS (10 - 100µg/mL) and sub fractions. Appropriate control and blanks were also prepared. The time zero-1 (T_{z1} plate) and -2 (T_{z2} plate) plates were fixed by addition of cold TCA (50% w/v, 50μL/well) before and after the addition of test agents in experimental plates. Followed by washing with distilled water after 30 minutes and left overnight for drying. Similarly, after 48h of incubation, experimental plates containing test substances were also fixed. All the plates (experimental, T_{Z1} and T_{z2}) were stained with sulforhodamine solution (100μL, 0.4% w/v in 1% acetic acid) for 30 min. The unbound stain was removed by washing (5x) with acetic acid (1%) and air-dried at room temperature. The protein bound stain was solubilized in tris base solution (pH 10.2, 100μL/well and 10mM) and absorbance was recorded at 515 nm. The absorbance values in the presence of the test agents were subtracted from blank values. If the absorbance value of the test well was greater than T_z plates, the percent growth was calculated as:

Cell growth (%) = $[(T-T_z)/(C-T_z)] \times 100$

However, if the absorbance value was less than Tz plates, the percent growth was calculated as:

Cell growth (%) = $[(T-T_z)/T_z] \times 100$

T_z and T represent the absorbance before and after the addition of test agents, respectively. T_z was calculated as the mean T_{z1} and T_{z2}. Control is represented by C.

The GI_{50} (concentration at which 50% of cells growth inhibit) and LC_{50} (concentration which killed 50% cells) was obtained from dose-response curves prepared by plotting the percentage of cell growth versus the concentrations of tested samples. All the experiments were repeated three times and conducted in triplicates as emphasized by the NCI, Frederick, USA laboratory.

The *in vitro* cytotoxicity assay was performed at cell culture facility of Panjwani Center for Molecular Medicine and Drug Research (PCMD), International Center for Chemical and Biological Sciences (ICCBS), University of Karachi.

STATISTICAL ANALYSIS

Three independent experiments data were presented as mean ± standard error of mean (SEM). IC_{50} values were obtained graphically using linear regression analysis (n = 3). All results were interpreted by one-way analysis of variance (ANOVA) by post hoc least significance difference (LSD) for the comparison among control and treated groups and Duncan range test to determine inter-

group differences. The accepted significance level for the tests was p <0.05 using statistical software package (SPSSv.12.0).

RESULTS

Growth inhibitory and cytotoxicity effect of S. aromaticum flower buds (Clove)

The methanol extract of clove exhibited dose dependent growth inhibitory effect against all three tested human cancer cell lines. It was most active in inhibiting cell growth of lung cancer cell lines (GI_{50} 110±2.9μg/mL) which was 1.2x and 1.6x better than uterine cervical cancer (GI_{50} 136±3.1μg/mL) and breast cancer (GI_{50} 180±3.7μg/mL) cell lines, respectively. Fraction SA-EAR was most active against HeLa (GI_{50} 30±1.1μg/mL) and it was 1.6 and 2.5 x more active against MCF-7 and H-460 cell lines, respectively. However, it produced cytotoxic effect (LC_{50} 90±3.7μg/mL) only against MCF-7. Fraction SA-PES-Fr-2 was most active (GI_{50} 36±3.1μg/mL) against MCF-7 and it was 1.4 x and 1.9x more active than HeLa and H-460 cancer cell lines. It produced–cytotoxic effect LC_{50}= 88 ± 3.4 μg/mL against HeLa and LC_{50}=86 ± 2.8 μg/mL against MCF-7. The fraction SA-EAS was only effective against HeLa cell line with (GI_{50} 36±3.4μg/mL). However fractions SA-PES, SA-AS, SA-MS, SA-MAQ were ineffective (table 1).

Analysis of active sub-fraction (SA-PES-Fr-2) by GC-MS

The GC total ion current (TIC) of active fraction (SA-PES-Fr-2) of petroleum ether soluble is shown in fig. 3. The chemical constituents of the fraction were identified by comparing their retention times and mass fragmentation patterns with the data of available reference samples and GC-MS spectral database. The percent composition of components in fraction was determined by computerized peak area measurements using an internal normalization method. Qualitative and quantitative data of present components are compiled in table 2 according

Table 2: Volatile constituents of active sub-fraction (SA-PES-Fr-2) of *S. aromaticum*

[a]Constituents	[b]Rt (min)	[c,d]RI	[e]Molecular weight	Formula	Percentage%
3-Acetoxy-3-hydroxypropionic acid, methyl ester	14.76	1115	162	$C_6H_{10}O_5$	0.18
Chavicol	18.44	1203	134	$C_9H_{10}O$	0.33
Eugenol	20.67	1392	164	$C_{10}H_{12}O_2$	85.34
Caryophyllene	21.86	1494	204	$C_{15}H_{24}$	2.62
1R,3Z,9s-4,11,11-Trimethyl-8-methylenebicyclo[7.2.0]undec-3-ene	22.47	1494	204	$C_{15}H_{24}$	0.11
α-Caryophyllene	22.57	1579	204	$C_{15}H_{24}$	0.48
Isoaromadendrene epoxide	23.83	1281	220	$C_{15}H_{24}O$	0.1
Acetyleugenol	24.01	1552	206	$C_{12}H_{14}O_3$	4.31
1H-Indene, 1-ethylideneoctahydro-7a-methyl-, cis-	24.61	1239	164	$C_{12}H_{20}$	0.29
Caryophyllenylalcohol	25.02	1677	222	$C_{15}H_{26}O$	0.21
β-Caryophyllene oxide	25.23	1507	220	$C_{15}H_{24}O$	1.71
1,5,5,8-Tetramethyl-12-oxabicyclo[9.1.0]dodeca-3,7-diene	25.74	1592	220	$C_{15}H_{24}O$	0.06
1,4-Methanoazulen-7-ol, decahydro-1,5,5,8a-tetramethyl-, [1s-(1α,3aβ,4α,7β,8aβ)]-	25.94	1593	222	$C_{15}H_{26}O$	0.05
Methyl 13,16-octadecadiynoate	27.65	2112	290	$C_{19}H_{30}O_2$	0.08
Benzyl Benzoate	28.61	1733	212	$C_{14}H_{12}O_2$	0.06
4,4,8-Trimethyltricyclo[6.3.1.0(1,5)]dodecane-2,9-diol	29.23	1840	238	$C_{15}H_{26}O_2$	0.37
2,6,6,10-Tetramethyl-undeca-8,10-diene-3,7-dione	29.77	1611	236	$C_{15}H_{24}O_2$	0.28
Palmitic acid methyl ester	31.96	1878	270	$C_{17}H_{34}O_2$	0.92
Linoleic acid methyl ester	38.23	2093	294	$C_{19}H_{34}O_2$	0.84
Oleic acid methyl ester	38.52	2085	296	$C_{19}H_{36}O_2$	0.32
Stearic acid methyl ester	39.93	2077	298	$C_{19}H_{38}O_2$	0.24
3-Methyl-2-pent-2-enyl-cyclopent-2-enone	57.60	1338	164	$C_{11}H_{16}O$	0.38
Benzyl stearate	61.07	2750	374	$C_{25}H_{42}O_2$	0.78
Ethyl iso-allocholate	68.39	3094	436	$C_{26}H_{44}O_5$	0.62

Notes: [a]Order of elution is given on column (ZB-5). [b]Compounds identified by their elution time. [c]Calculated retention indices of compounds. [d]Compounds identified by comparing with retention indices from the literature available in NIST database. [e]MS=identification by comparing EI mass spectrum with NIST mass spectral data base; RI=identification by retention indices with literature data. Rt=elution time of compounds from column.

to their elution order on Zebron^{TM}ZB-5MS fused silica capillary column. Fraction showed the presence of different chemical constituents mainly consisting of oxygenated mono and sesquiterpene, hydrocarbons and fatty acid derivatives. The major organic chemical compounds identified in the fraction were eugenol (85.34%), acetyleugenol (4.31%), caryophyllene (2.62%), *β*-caryophyllene oxide (1.71%), palmitic acid methylester (0.92%), linoleic acid methylester (0.84%), benzyl stearate (0.78%), *α*-caryophyllene (0.48%), caryophyllenyl alcohol (0.21%) and chavicol (0.33%). Some minor chemical constituents were also identified in the active fraction (SA-PES-Fr-2) of petroleum ether soluble (SA-PES).

DISCUSSION

In the present study the GC-MS analysis of active petroleum ether soluble sub fraction (SA-PES-Fr-2) revealed the presence of some important pharmacologically active metabolites in high concentrations such as phenyl propanoids: eugenol, acetyleugenol and chavicol; saturated fatty acid methyl ester: palmitic acid methylester; unsaturated fatty acid esters: linoleic acid methylester, benzyl stearate; sesquiterpenes: *β*-caryophyllene and *β*-caryophyllene oxide, α-caryophylleneandcaryophyllenyl alcohol. Eugenol is known to possess cytotoxic activity against colon cancer (SNU-C5), leukemia HL-60 and liver (HepG2) (Carvalho *et al*., 2015; Kim *et al.*, 2014; Yoo *et al*., 2005). *β*-Caryophyllene oxide is sensitive towards multiple myeloma (Lima *et al*., 2012). Both saturated and unsaturated fatty acids and their methyl esters are well known cytotoxic compounds inducing apoptosis in several human cancer cell lines (Carrill *et al.*, 2012; Wu *et al.*, 2005; Yoo *et al*., 2007). Further, *α*-caryophyllene also possesses cytotoxic activity (Loizzo *et al*., 2007). In addition, the growth inhibition activity of residue obtained from main ethyl acetate phase (SA-EAR) and its ethyl acetate soluble fraction (SA-EAS) against HeLa cancer cells line (GI_{50} 30±1.1*μg*/mL and 36±3.4*μg*/mL, respectively) may be due to the presence of anticancer triterpene oleanolic acid (Rangari and Banarase, 2010; Zhu *et al*., 2015). Furthermore, growth inhibition activity (GI_{50} 36±3.1μg/mL) of petroleum ether soluble sub fraction (SA-PES-Fr-2) against MCF-7 cell line may be due to the presence of eugenol (Anita *et al*., 2012; Carvalho *et al*., 2015; C.-B. Yoo *et al*., 2005) which is the main constituent of this fraction (*loc. cit.*).

These findings suggest that the growth inhibition of *S. aromaticum* flower buds is due to the presence of a variety of metabolites and necessitates further scientific studies. This will open a new direction towards obtaining potential anticancer agents from clove.

CONCLUSION

In conclusion, our findings demonstrated that flower buds of *S. aromaticum* possesses growth inhibitory properties against different human cancer cell lines supporting its use in traditional system of medicine for treatment of cancer. In future, the metabolites from clove need to be further investigated to elucidate their mechanism of action.

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