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Antimicrobial activity and cytotoxicity of *Theobroma cacao* extracts

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ABSTRACT

The cocoa leaf (CL), seeds (CS) and pod (CP) were studied for the estimation of medicinal properties that they contain and the focus was screening for anticancer ability. Extraction of the samples in Soxhlet apparatus was done with methanol as the solvent followed by phytochemical analysis, antimicrobial activity, antioxidant assay and anticancer activity. Thin Layer Chromatography (TLC) showed the number of compounds in hexane:ethyl acetate: methanol solvent system for cocoa pod and chloroform:methanol solvent for cocoa leaf under UV illuminator. The antimicrobial assay by agar well diffusion method of cocoa seeds showed zone of inhibition against pathogens Serratia marcescens, Staphylococcus aureus, Salmonella sp. and Shigella dysenteriae. Further, antioxidant assay was performed using DPPH radical scavenging assay. The sample was then tested for cytotoxicity assay against MG63 osteosarcoma cell lines and active compounds were identified by doing Gas chromatography and Mass spectroscopy (GCMS).

Keywords: Soxhlet extraction, antimicrobial, antioxidant, phytochemical screening, MG63 cell lines.

INTRODUCTION

Theobroma cacao, popularly known as Cocoa, is an evergreen tree belonging to the family *Malvaceae.* The sequence of the cacao genome identified 28,798 protein-coding genes, about 20% of which consist of transposable elements. Among them many were identified as coding for flavonoids, aromatic terpenoids, theobromine and many other metabolites. A relatively high proportion of genes code for polyphenols, which constitute up to 8% of cocoa pods dry weight. The fermented, dried seeds are considered as aphrodisiac, and its unique chemical composition promotes a certain amorous and energetic brain activity which is attributed to the psychoactive alkaloids theobromine and caffeine. Infusions of leaves are used as cardio tonic and diuretic. The seeds are high in copper, sulphur and vitamin C. Two flavonoids in particular, catechin and epicatchin are found in extremely high amount in cocoa and have copious amount of research supporting their wealth benefits. The most well-known chemical found in cocoa is phenyethylamine (PEA) which helps the body to produce opium-like compounds, commonly known as endorphin that boosts levels of the neurotransmitter dopamine. Cocoa bean has abundant deposit of polyphenols. Total polyphenol content of cocoa is significantly higher than blueberry, cranberry, pomegranate and consequently anti-oxidant activity is much higher than other. The antimicrobial activity of cocoa polyphenols against Gram positive and Gram negative by agar diffusion inhibition method showed this property of cocoa polyphenols in a new discussion [1]. Here methanol and acetone was found to be better solvents for extraction of antimicrobial active substances compared to water.

The anti-oxidant capacity of water and ethanolic extracts from cocoa pods showed amazing results. The extract made from water showed the higher value of anti-oxidant activity based on β-carotene bleaching assay, while the extract made from ethanol showed the highest scavenging results and ferric reducing activities [2], the ability to scavenge superoxide radicals and hydroxyls radicals, reduce peroxyl radicals and inhibit lipid peroxidation [3]. Cocoa help oxygenate the brain and promote detoxification of industrial chemical in the body. Cocoa has an

important, immune stimulating effect and can be used adjunctively to treat cancer, the centre of research in this paper.

MATERIALS AND METHODS

Sample Collection and Preparation

The cocoa seeds, leaves and pods were explanted from a cocoa plant in nursery of VIT University, Vellore, Tamil Nadu, India. The samples were collected in sterile polyethene bags and transported to the laboratory. Further, they were washed under tap water for 10-15 min followed by sterile distilled water for 5-10 min. Later the samples were sun dried for 3-4 days until a constant weight and stored in airtight containers.

Soxhlet Extraction

The crude samples of Cocoa leaf, seed and pods were collected and coarse powder was made using a mixer grinder. 30g of the powdered sample was subjected to extraction with Soxhlet apparatus using methanol. The process continued for 4-5 h followed by filtration with Whatman filter paper no. 1. The filtered extract was dried in vaccum drier and the resulting extract was stored at 4℃ for further analysis.

Thin Layer Chromatography (TLC)

TLC was performed and the analysis was made on silica gel bed solidified on aluminium sheath. Four different solvent systems were used for the analysis of the extracts. The first solvent was prepared by adding hexane, ethyl acetate and methanol in the ratio of 4:2:2. The second solvent was prepared by mixing hexane, formic acid and glacial acetic acid in 4:2:2 ratio. Third solvent system was prepared using hexane, acetone and ethyl acetate mixed in the ratio of 4:2:2 while the last solvent was prepared by mixing chloroform with methanol in 8:2 ratio. Solvent front was drawn on the pre-coated (silica gel 60) TLC sheet, with a pencil marked a line across the plate 10mm (=1cm) from one end. The four freshly prepared solvents were poured into 50ml beakers, labelled respectively and was covered with aluminium foil. A capillary tube was used to make a spot of the sample extract on the TLC sheet and the TLC sheet was placed inside the beaker upright. It was made sure that the spot was above the solvent. Care was taken that TLC was not touching the sides of the beaker.

As the solvent reached the marked solvent front line, the TLC plates were removed and the end points of the visible pigments were marked respectively for each prepared solvents. The plates were then air dried and from the distance of the solvent moved, we can quantify the rate of migration was quatified for the compound using the ratio referred to as the Rf value.

$$R_f \text{ value} = \frac{\text{Distance travelled by solute front}}{\text{Distance travelled by solvent front}}$$

The TLC plates were then visualized under UV Light source, using UV Trans-illuminator.

Phytochemical Screening

The filtered crude extract of cocoa was partitioned by sequential extractions of ethyl acetate, n-hexane, chloroform and 70% ethanol after being concentrated under reduced pressure. These formed fractions which were calculated by phytochemical qualitative reactions for common secondary metabolites of cocoa plant, then screened for carbohydrates, alkaloids, flavonoids, saponins, tannins and phenolic acids. The colour intensity of the precipitate which was formed was used as analytical test controls.

Test for carbohydrates (Fehling's test): About 1ml of Fehling A and Fehling B solution were added to the extract. Then it was allowed to heat for 30 min and observed for the formation of brick red color which indicated the presence of carbohydrates.

Test for alkaloids (Wagner's test): A small amount of extract was taken in a test tube and 3-5 drops of Wagner's reagent (1.27g of iodine and 2g of potassium iodide in 100ml of water) was added. Formation of reddish brown precipitate or coloration indicated the presence of alkaloids in the extract.

Test for saponins (Foam test): About 0.1g of sample was mixed with 5ml of distilled water and allowed to boil. Then the mixture was filtered and 2 drops of olive oil was added in 1ml of filtrate. The mixture was shaken and formation of emulsion and froth was observed. The 1ml filtrate was diluted by adding upto 4ml of distilled water. The mixture was shaken vigorously and observed for a stable froth.

Test for flavonoids: The filtrate was prepared by boiling the mixture of 0.5g of sample and 10ml of ethyl acetate for 1 min. Then the mixture was filtered and 4ml of filtrate was shaken with 1ml of 1% ammonium chloride solution. Formation of yellow color in the presence of ammonium solution indicated the presence of flavonoids.

Test for phenols (Ferric chloride test): About 1ml of extract was mixed with 1ml of distilled water and warmed. To this 2ml of ferric chloride solution was added. Formation of green or blue color confirmd the presence of phenols.

Test for tannins: 5grams of the dried and powdered sample was boiled in 20 ml of water in a test tube with the aid of a water bath and was filtered. Then add a few drops of ferric chloride to the test tube. Appearance of brownish green or bluish black coloration indicated the presence of tannins.

Anti-Microbial Assay

On Mueller-Hinton agar each bacterial inoculum was swab streaked that has been previously prepared by inoculating pathogens *Staphylococcus aureus*, *Eschericha coli*, *Salmonella* sp., *Shigella dysenteriae*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Enterobacter* sp., *Pseudomonas aeruginosa* and *Proteus mirabilis* into nutrient broth with overnight incubation, which were obtained from Microbial Biotechnology Lab, VIT University. The wells were cut into agar plates and different concentrations of sample extract solutions were placed in the wells, i.e. 25µl, 50µl, 75µl, 100µl (25 mg/ml) and 100µl of methanol was added to one well as control. Plates were then incubated for 24h at 35± 0.5°C. The clear zone of inhibition was observed and measured in mm.

Anti-Oxidant Assay

Each extracts with concentration 25 mg/ml (100 µL) was pipetted into 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution concentration 100 µL (1:1) to initiate the reaction. The absorbance was read at wavelength 517 nm after 15 minutes incubation using UV-Vis spectrophotometer. Methanol concentration taken was 2900 µLwas used as a blank and DPPH solution as standard. Analysis was done in duplication for standard and each extracts. All measurement procedures were performed in dark room. Antioxidant activity of each extracts was determined based on the reduction of DPPH absorbance by calculating percentage of antioxidant activity.

A volume of 300µl was used as 100% of the anti-oxidant activity as calculated by the given equation, where "A" represents each absorbance value:

$$\text{Antioxidant activity (\%)} = \frac{(A_{standard} - A_{test)}}{(A_{standard})} \times 100$$

Gas Chromatography – Mass Spectrometry (Gcms)

The carrier gas was chemically inert and He (120 kPa) was taken. The Injection was Split and with the help of Microsyringe 1 µL of sample extract was injected and the temperature was kept at 250°C. Inert Cap Pure-Wax Column was used (0.25mmI.D x 30m df= 0.25µm) and the column temperature was set at 40°C first then dropped down to 4°C per min and again increased to 250°C and was kept for 5 mins. The detection was done by MS scan (m/z; 55-400) was used to detect the sample.

Anti-Cancer Activity

The human osteosarcoma cell line (MG 63) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). The cells were maintained at 37^0C, 5% CO2, 95% air and 100% relative humidity. The cytotoxicity was determined using MTT assay [4]. MG63 cells were seeded at a density of 1×10^4 cells/well in 100 µl of cell culture medium and them placed in 96 well microtitre plate. After 48 hours of incubation, 15µl of MTT (5mg/ml) was dissolved in phosphate buffer saline (PBS), was micro-pipetted to each well and again incubated at 37°C for 4 h. The medium containing MTT was then removed and the newly formed Formazan crystals were solubilized in 100µl of DMSO. The absorbance was measured at 570 nm using a micro plate reader [5].

The cell viability percentage was then calculated with respect to control as follows:

$$\text{Cell viability (\%)} = \frac{[A]_{Test} \times 100}{[A]_{Control}}$$

The cell inhibitionpercentage was determined using the following formula:

$$\text{Cell Inhibition (\%)} = \frac{[100 - \text{Abs (sample)}] \times 100}{\text{Abs (control)}}$$

Nonlinear regression graph was plotted between Cell inhibition percentage and Log concentration and IC50 was determined with the help of GraphPad Prism software.

RESULTS AND DISCUSSION

Phytochemical Analysis

Preliminary phytochemical screening of all the three extracts showed the presence of different phytochemical classes as shown in table 1. The presence of these phytochemicals active compounds identified are known to have beneficial importance in medicinal properties used in pharmaceutical and therapeutic purposes. Saponins and flavonoids were present in all the extracts. Saponins are mild detergent with therapeutic effects such as anticancer, hypercholestrolemic, antioxidant, etc. [6]. However, flavonoids are also known for a wide range of biological activities like antimicrobial, analgesic, antiallergic, cytostatic and anti-inflammatory. Also tannins have antiviral, antibacterial, anticancer properties. They are also able to inhibit HIV replication , sensitivity and diuretic [7].

Table 1. Phytochemical screening of Cocoa extracts

S. No.	Solvents	Extract	Solute front (cm)	Solvent front (cm)	R_f
1	hexane:ethyl acetate:methanol	pods	4.7	5.0	0.94
		seeds	4.8	5.2	0.92
2	hexane:formic acid:glatial acetic acid	leaves	2.8	5.1	0.55
3	hexane:acetone:ethyl acetate	pods	2.9	4.2	0.69
		seeds	2.9	4.3	0.67
		leaves	2.8	4.2	0.67
4	chloform:methanol	leaves	3.4	3.6	0.94

Thin Layer Chromatography

The TLC was used to identify each components of the extracts by its characteristic R_f value. For solvent 1 we observed two spots for pods and seeds with a R_f value of 0.94 and 0.92 respectively. While from solvent 2 we observed only one spot for leaves with a R_f value of 0.55, for solvent 3 we observed 3three spots for pods, seeds as well as leaves with a R_f value of 0.69, 0.67 and 0.67 respectively. For solvent 4 we observed only a single spot for leaves with a R_f value of 0.94.

Anti-Microbial Assay

Antimicrobial assay was done by measuring the zone of inhibition for the extracts of cocoa samples against nine pathogenic strains. Cacao seeds extract was able to inhibit four pathogenic strains which include *S. aereus*, *Salmonella* sp., *K. pneumoniae* and *S. marcescens* showing a zone of inhibition range of 12-17 mm in diameter. Similarly, zone of inhibition was observed against *S. aureus*, *S. dysenteriae* and *K. pneumoniae* when cacao leaves extract was placed in the wells. There was a maximum zone of 23 mm against *K. pneumoniae* suggesting potential antimicrobial effect which makes it an ideal drug. The agar well diffusion assay of cacao pod extract depicted the inhibition against four different clinical pathogens namely, *S. aureus*, *Salmonella* sp., *K. pneumoniae* and *P. aeruginosa*. Different concentrations of the extract showed different level of inhibition with a highest of 17 mm diameter. The results with all the zones and concentrations for all the three extracts are given in table 2.

Table 2. Antimicrobial activity of Cocoa extracts

S. No.	Pathogen	Zone of Inibition (mm)								
		Seeds extract (µl)			Leaves extract (µl)			Pods extract (µl)		
		50	75	100	50	75	100	50	75	100
1	*Staphylococcus aureus*	13	16	17	20	22	23	12	14	15
2	*Eschericha coli*	-	-	-	-	-	-	-	-	-
3	*Salmonella* sp.	18	19	21	-	-	-	-	13	16
4	*Shigella dysenteriae*	-	13	18	-	15	17	-	-	-
5	*Klebsiella pneumonia*	-	14	19	21	23	23	-	12	14
6	*Serratia marcescens*	-	11	17	-	-	-	-	-	-
7	*Enterobacter sp.*	-	-	-	-	-	-	-	-	-
8	*Pseudomonas aeruginosa*	-	-	-	-	-	-	-	10	13
9	*Proteus mirabilis*	-	-	-	-	-	-	-	-	-

Antioxidant Activity (%) Assay:

Antioxidant activity was evaluated by DPPH method. The total antioxidant property of the extracts refers to the free radical scavenging capability of the components present in the extract. DPPH gives purple coloration to the reaction mixture but if any antioxidant is present, there is a color change from purple to yellow showing decolorization of DPPH to form Maximum scavenging was observed in the pod extract as shown in table 3. The reducing activity of the extracts followed the order pods >leaves >seeds, which could be due to high amount of reducing agents in the pods extract. The reducing properties are generally associated with the presence of reducing agents with antioxidant action, by breaking the free radical chain through donating a hydrogen atom [8]. Tannin is a group of polyphenolic phytochemicals, which is believed to be responsible for dietary astringency and is widely distributed in cocoa. A number of studies have shown the correlation of the antioxidant activity of extracts with the phenolic content of the

extract. The antioxidant property of the phenolics is mainly due to their redox potential which allows them to act as a good reducing agent, hydrogen donor and single oxygen quenchers [9].

Table 3. Antioxidant activity of Cocoa extract

S. No.	Sample	OD at 517 nm	% Scavenging Activity
1	Standard (10mg/ml)	0.802	_
2	Seeds extract	0.306	61.85
3	Leaves extract	0.231	71.20
4	Pods extract	0.210	73.82

Gas Chromatography – Mass Spectrometry (Gcms)

The chromatogram of seed extracts depicts the presence of predominantly caffeine and theobromine in the seed extract (fig. 1). Some traces of isoquinolone were also found in the extract. In the leaves extract esters and ketones (fig. 2) were predominant while pods extract showed the presence of papeveroline (fig. 3). The peaks procured from the mass spectrometric unit were compared with the Nist library data to obtain the best match and structure of the compounds which could be the reason of the antimicrobial activity of the extracts.

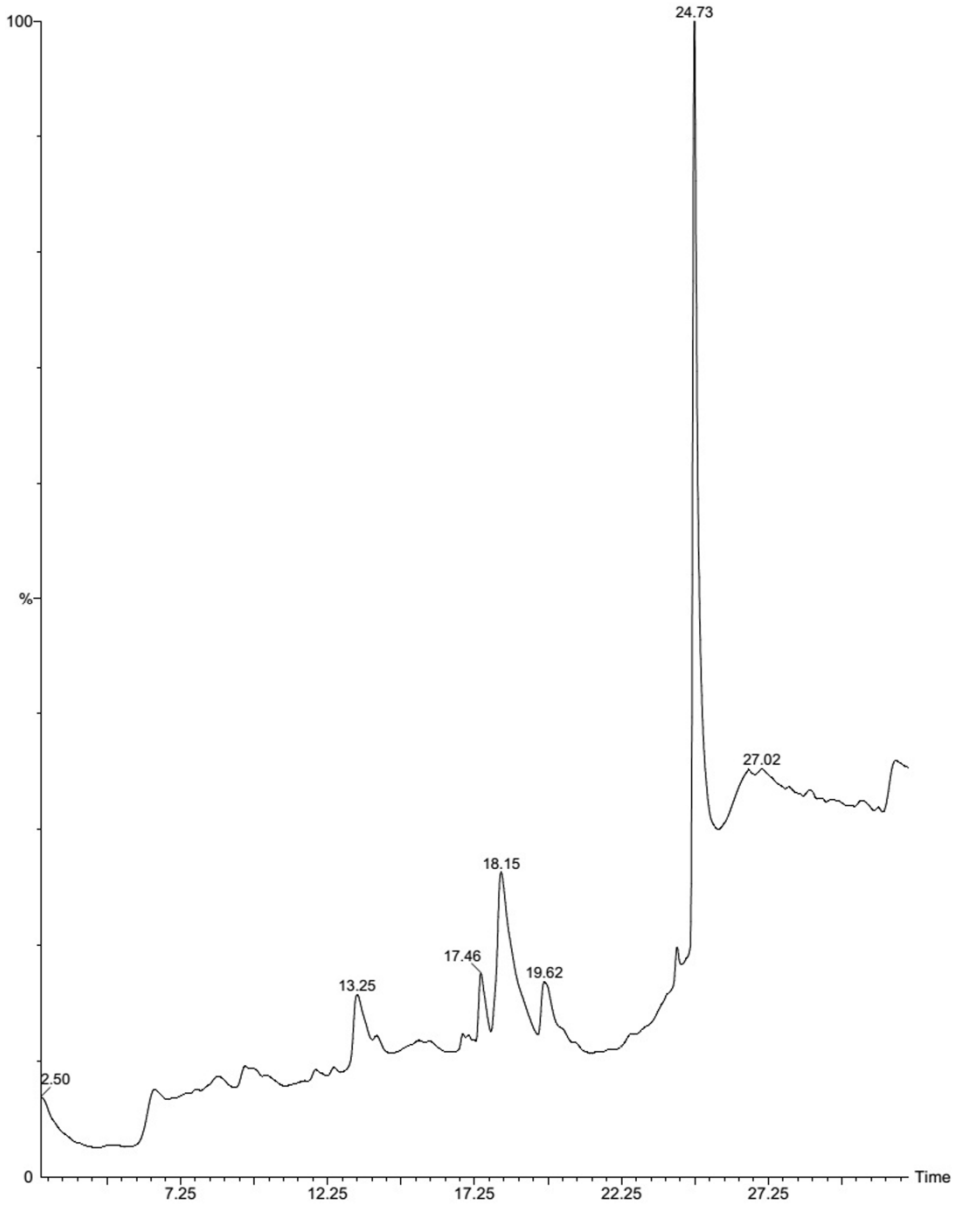

Fig. 1 GC-MS analysis of Cocoa seeds extract

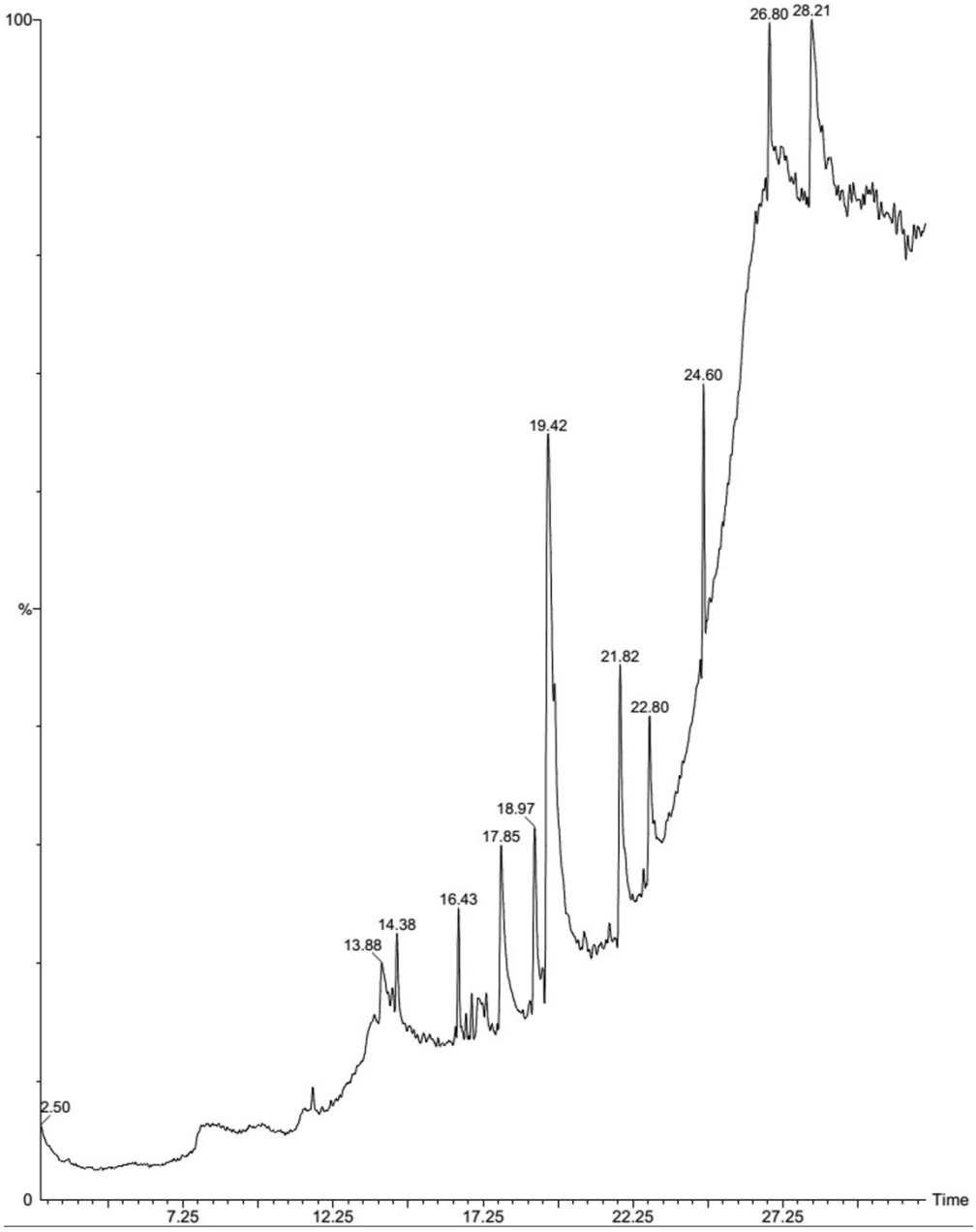

Fig. 2 GC-MS analysis of Cocoa leaves extract

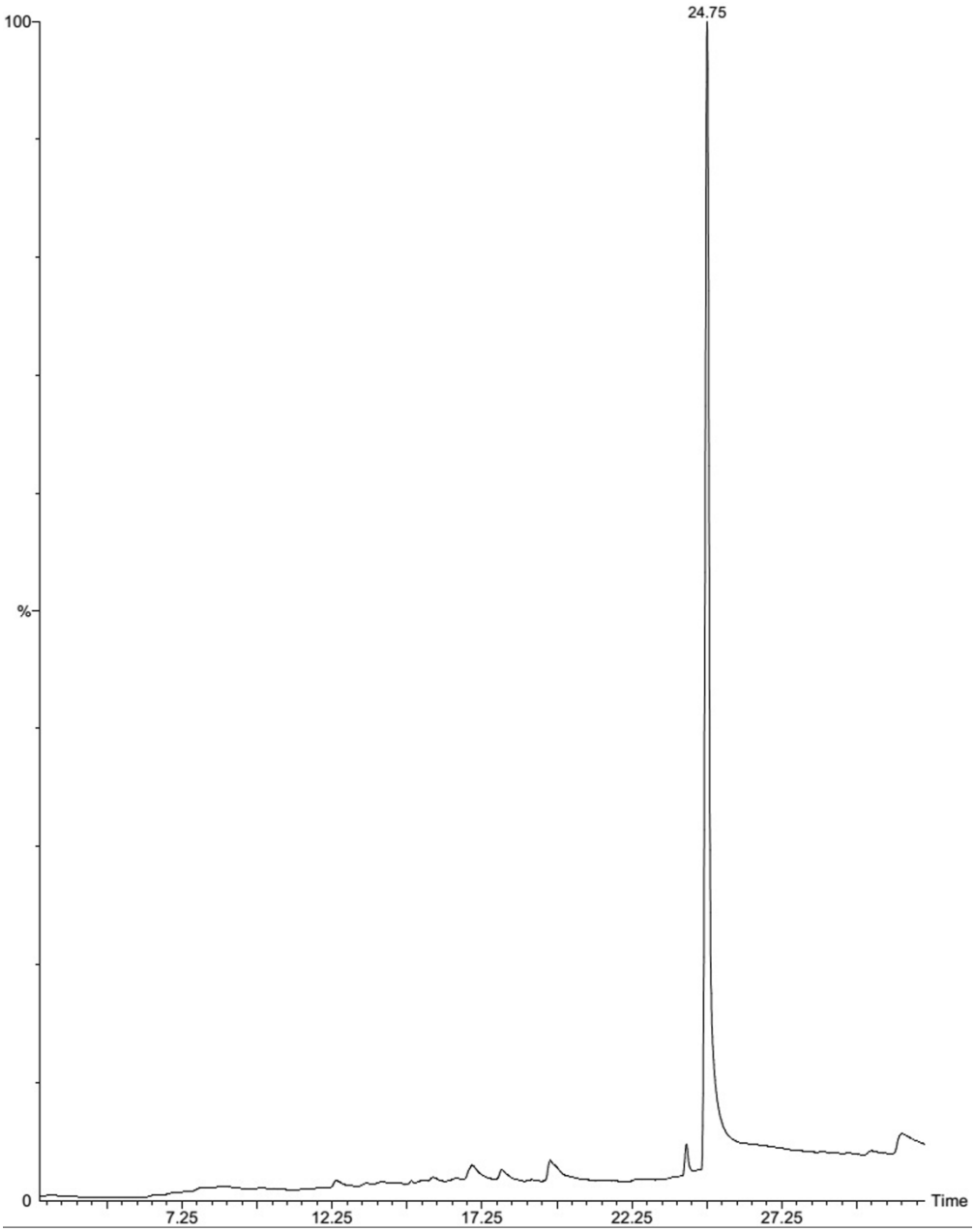

Fig. 3 GC-MS analysis of Cocoa pods extract

Cytotoxicity Assay

Since the extracts showed good amount of antioxidant activity, they were further processed for anticancer activity against MG63 osteosarcoma cell lines. All the three extracts showed deterioration in the cell lines but among all the three extracts pods extract showed maximum inhibition of the cancer cell lines. Based on the anticancer screening using MTT assay illustrated in figure 4, with an increase in the concentration of the extract, the inhibition was also directly proportional causing the cancer cell death in an increasing fashion. There was a moderate activity against the osteosarcoma cell lines, which was confirmed with the MTT assay which is a typical method commonly used in determination of anticancer activities. Thus the exposure of cancer cells to cocoa extracts induced a significant reduction in the conversion of MTT which means a cellular disintegration and cytotoxicity reflected from the parallel dose and time dependent decrease of the absorbance measured [10]. As the cytotoxicity was observed more pronounced in cocoa pod extract, the deterioration in the can cell lines is depicted in figure 4. MG63 osteosarcoma cell lines were chosen with respect to its occurrence and fatality to the patient. There has been no reports on activity of of cocoa extracts against MG63 cell lines, thus the present work was a trial attempted to check its activity against MG63.

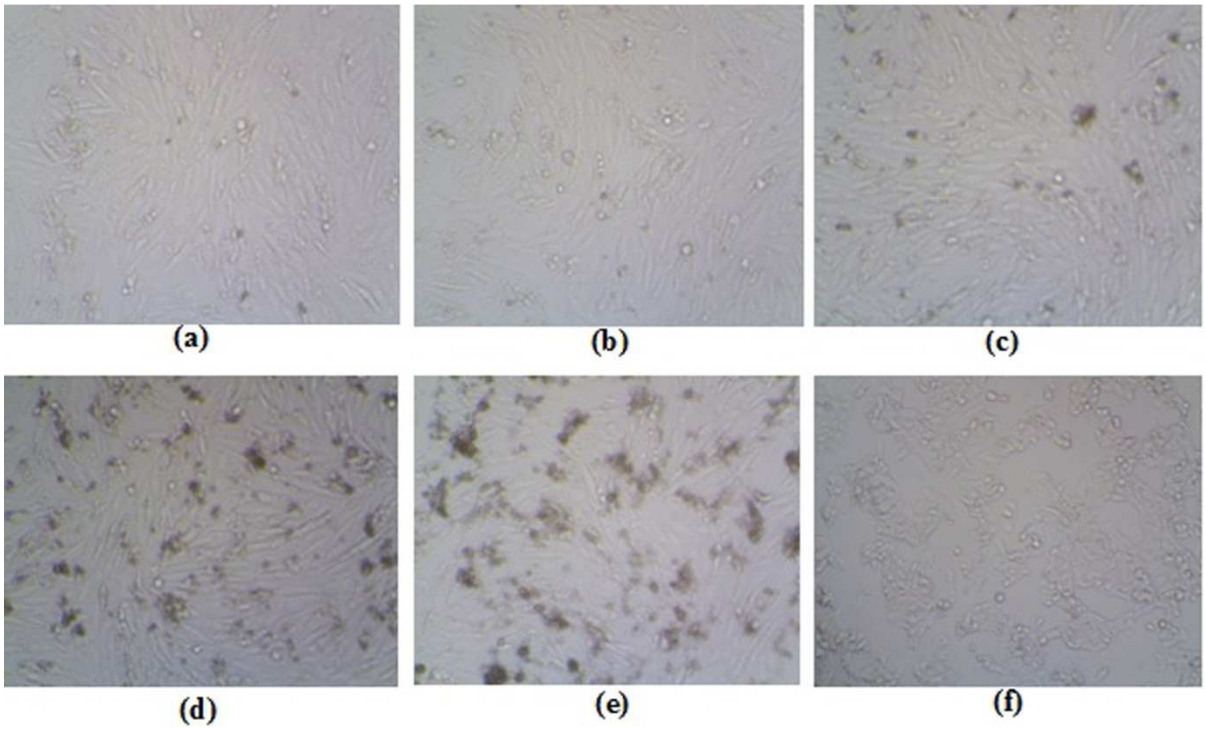

Fig. 4 Cytotoxicity result of Cocoa pod extract at different concentrations (µg /ml) (a) 12.5 (b) 25 (c) 50 (d) 100 (e) 200 (f) control

CONCLUSION

Plant as a source of naturally occurring bioactive compounds have shown remarkable potential in pharmaceutical and therapeutic area. They are a great hub of secondary metabolites with enormous biological prospective. The work done here shows the potential of the cocao plant extracts against pathogenic bacteria and also there is scope of some cytotoxic activity. The extracts contain certain compounds which can be the reason of the biological activities present in the extracts. Thus, there is a great chance of them to be used as antimicrobial drug with further purification and proper processing.

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