ANTICANCER ACTIVITY OF VARIOUS KAN-ZAW EXTRACTS ON HUMAN CERVICAL CANCER CELLS (HELA)*

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Abstract

Payena paralleloneura Kurz. also called Kan-zaw is a large evergreen tree belonging to the family Sapotaceae which is widely used in the treatment of various cancer and different ailments. In the present work the fatty acid analysis of the Kan-zaw seed oil contains approximately >70% α-eleostearic acid and 3.25 % β-eleostearic acid an unusual conjugated fatty acid that imparts a potent anticancer application and industrially important drying qualities to Kan-zaw oil. The present study also investigated cytotoxic potential of various extracts of Kan-zaw seeds using ethanol, methanol, acetone, chloroform and commercial Kan-zaw oil against human cervical cancer cell line (HeLa) by MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The various extracts of Kan-zaw seeds (ethanol, methanol, acetone, chloroform) and commercial Kan-zaw oil were effective towards tested cell line, with inhibition and they were found that with the highest 87.14%, 81.56%, 87.48%, 87.99% and 88.32% respectively. The *Payena paralleloneura* Kurz. (Kan-zaw) extracts and oil have shown significant anticancer activity on HeLa cells.

Keywords: *Payena paralleloneura* Kurz., Fatty Acid analysis, Anticancer activity

Introduction

Conjugated fatty acids are naturally occurring compounds that have specialized uses in nutraceutical and industrial applications. For example, conjugated linoleic acid (CLA) is a potent anticancer compound present in foods derived from ruminant animals (Belury, 2002). Conjugated fatty acids such as α-eleostearic acid have recently shown promise for anticancer applications (Kohno *et al*., 2002), as well as serum lipid lowering effects in mammals (Koba *et al*., 2002). Oils containing α-eleostearic acid may also be used for industrial drying applications. A third mechanism for generating

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conjugated fatty acids, which is typical of higher plants, involves fatty acid oxidation and bond rearrangement. For example, radiolabeling studies with developing bitter gourd seeds revealed that linoleic acid was modified at the position to produce α-eleostearic acid, Conjugated linolenic acids (CLNs), lack the methylene groups found between the double bonds of linolenic acid. Oils rich in conjugated linolenic acids (CLNs) are important medicinally as a source of nutraceuticals and industrially as drying agents in paints, inks, and varnishes (Biermann *et al*., 2011). α-Eleostearic (α-ESA) acid is the most widespread CLN. Tung *(Aleurites fordii)* and bitter gourd (*Momordica charantia*) seeds are rich source of α-ESA. Other geometrical isomers of α-ESA that are found in nature are punicic acid, catalpic acid, and β-ESA. Punicic acid is found in pomegranate (*Punica granatum*) and snakegourd (*Trichosanthes kirilowii*) seeds, catalpic acid is present in catalpa (*Catalpa bignonoides* and *Catalpa ovata*) seeds, and β-ESA is present in pomegranate, bitter gourd, and catalpa seeds (Joh, *et al.,* 1995; Ozgul, 2005). Additional positional isoforms of α-ESA known are calendic acid and jacaric acid and are present in pot marigold (*Calendula officinalis*) and jacaranda (*Jacaranda mimosifiola*) seeds, respectively (McClean and Clark 1956; Hopkins and Chisholm 1968).

Cervical cancer constitutes the second most common cancer in women. This is due to the infection with Human Papilloma Virus (HPV), notably type 16 and 18 virus (Alvarez - Salas and DiPaolo, 2007).

Natural products are being tested for the treatment but these are yet to prove their efficacy in preclinical and clinical studies. Some of the food plants are believed to be an important source of nutrition as well as chemical substances having potential of therapeutic effects. These plants are effective source of both traditional and modern medicines and are genuinely useful for primary healthcare. Plants have been rich source of medicine because they produce wide range array of bioactive molecule (Agharkar, 1991).

According to local people and traditional practitioners which they used its fixed oil from the seeds as major remedy traditional medicine for the

treatment of breast, cervical, ovarian and various cancers, anti-peptic ulcer, paralysis, bronchitis, rash, chest pain, injury, sores and various other ailments.

These are used for primary healthcare in rural areas in my countries. This tree known under the name of Kan-zaw, produces Kan-zaw oil with high medicinal value. It is highly regarded as a universal panacea in the ayurvedic medicine and large evergreen tree distributed in only Tanintharyi region of Myanmar. The Kan-zaw seeds have not been previously systematic studies on the anticancer activity of Kan-zaw seeds. The main aims of analyzing crude plant extracts are agents for direct use as drugs that can be used as lead substances in the preparation synthetic drugs. There are two main strategies for the selection of plants species in anticancer drug discovery: the first one contain random screening and ethno medical knowledge. The second approach includes plants used in organize traditional medical systems like herbalism and folklore. The search for new anti-cancer drugs is one of the most prominent research areas of natural products. In the present study, the anticancer activity of Kan-zaw seeds were investigated.

Materials and Methods

Fatty acid analysis of Kan-zaw seeds (FAME analysis)

The kernels of the Kan-zaw seeds were dried and crushed into powder in a motor and pestle. Oil was extracted from around 50 mg powder in a glass tube (2cm x 10cm) with a screw cap. To this tube, 3 ml of Hexane was added and vortexed for 10 sec followed by an extraction process under a ultrasonic cleaner (25K Hz) for 1.5 hr. Next, to the sample 400 µl of KOH (5M)/Methanol was added to esterify fatty acid under the ultrasonic for additional 10 min. After cooling to room temperature, 200 µg of methyl heptadecenoate (C 17:1) was added as an internal standard. The mixture was vortexed for 10 sec before centrifugation at 5000 rpm for 5 min. The compounds of fatty acid methyl esters (FAMEs) in the upper organic phase were removed and the residual mixture was extracted for FAMEs with additional 3 ml of Hexane. The pooled FAME extracts were evaporated under nitrogen and then dissolved in 500 µl of Hexane for gas chromatograph

analysis (GC; Agilent 7820A, CA) with a flame ionization detector (FID) on a DB-23 column (30 m by 0.25 mm id., 0.25 µm film; Agilent, CA). The GC conditions were split mode injection (1:20), injector and flame ionization detector temperature, 270°C and 280°C; the oven condition was 80°C for 3 min, with a ramp to 170°C for 10 min with 10°C increments per minute, then increasing at 5°C/min to 220°C. FAME compounds were identified by calibration with a standard (NU-CHEK, USA), a mixture for 37 known FAMEs.

The oil content was then calculated from the famous: Percent oil by dry weight = (100 x total peak area x 0.2 mg/ peak area of internal standard)/mg tissue, where 0.2 mg is the amount of internal standard used per sample. The composition percentage of a single FAME was reflected by a percentage of the peak area of this FAME in total peak area of all FAMEs (Wychen, 2015).

Various extraction of Kan-zaw seeds by using rotary evaporator

The various extraction of Kan-zaw seeds were determined according to (The British Pharmacopoeia, 1965) as follows. Forty gm of Kan-zaw seeds powdered was soaked with 250 ml of ethanol in a closed flask for 72 h and kept over three nights. The mixture was filtered rapidly taking precautions against loss of alcohol and then the filtrate was put into round bottle flask and extracted by using rotary evaporator. The extract was obtained by drying the concentrated pooled extract under vacuum (Suthar and Mulani, 2008).

The above procedure was extracted with methanol, acetone and chloroform instead of ethanol. Commercial Kan-zaw oil for analyses were purchased from the market.

***In vitro* evaluation of anticancer activity by MTT assay**

Cell Culture

The human cervical cancer cell line (HeLa) was obtained from National Centre for Cell Sciences (NCCS), Pune and grown in Eagles minimum essential medium (EMEM) containing 10% Fetal Bovine Serum (FBS). All cells were maintained at 37º C, 5% CO2, 95% air and 100% relative humidity.

Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

Cell Treatment

The monolayer cells were detached with trypsin-ethylene diamine tetra acetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of

1 x 10^5cells /ml. 100 µl per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37ºC, 5% CO2, 95% air and 100% relative humidity.

After 24 h the cells were treated with serial concentration of the test samples. They were initially dissolved in Dimethyl sulfoxide (DMSO) and using the 250 mg/l concentration various extract four serial dilutions of the extract of 500 µl each was prepared DMSO to get the concentration of the extract from 125-250 mg/l as indicated in Table 2, 3, 4, 5 and 6. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulted the required final sample concentrations. Following the treatment with various extract of Kan-zaw seeds (ethanol, methanol, acetone, chloroform) and commercial Kan-zaw oil, the plates were incubated for an additional 48 h at 37ºC 5% CO2, 95% air and 100% relative humidity. The medium without samples were served as control and triplicate was maintained for all concentrations (Slater *et al*, 1963; Alley, *et al*, 1988 and Van de Loosdrecht, 1994).

MTT Assay

After 48 h of incubation, 15 µl of MTT (5 g/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37ºC for 4 hr. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 µl of DMSO and then measured the absorbance at 490 nm using microplate reader. The same procedure was carried out for the

extraction with different solvents. The % cell inhibition was determined using the following formula (Jack, 2005).

Percentage cell inhibition = 100 - Abs (Sample) / Abs (Control) x 100.

Abs (Sample) = Absorbance value of sample

Abs (Control) = Absorbance value of control

Results and Discussion

Fatty acid analysis of Kan-zaw seeds

In this research, the mass spectrum of the FAME corresponding to peak a exhibited a molecular ion at *m/z* = 261, characteristic of a 16:2 methyl ester, where as the spectrum of a prominent molecular ion at *m/z* = 292, indicative of an 18:3 methyl ester. α-Eleostearic acid (α-ESA) is the most widespread CLN Fig. 1. The methyl esters of palmitic (16:0), stearic (18:0), oleic (18:1), and linoleic (18:2) acids as shown in Fig. 2. This is the agreement with the findings of John *et al*. (2002) who reported the results. Conjugated fatty acids such as α-eleostearic acid have recently shown promise for anticancer applications and industrially as drying agents in paints, inks and varnishes (Koba *et al*., 2002 and Kohno *et al*., 2002).

The GC chromatogram of FAME derived from the Kan-zaw oil (*Payena parallelonеura* Kurz.) tree contains approximately >70% α-eleostearic acid and β-eleostearic acid methyl esters also presented in Fig. 3. Joh, *et al*., (1995), Ozgul, (2005) and Richa Rawat *et al*. (2012) stated that Tung (*Aleurites fordii*) and bitter gourd (*Momordica charantia*) seeds are rich source of α-ESA and accumulate >80% and >60% of α-ESA, respectively. β-ESA is present in pomegranate, bitter gourd, and catalpa (*Catalpa bignonoides* and *Catalpa ovata*) seeds.

The fatty acid composition of Kan-zaw seeds is presented in (Fig. 3 and Table 1). It comprises of palmitic acid (C16:0) 3.41%, margaric acid (C17:0) 0.23%, stearic acid (C18:0) 2.45%, oleic acid (C18:1) 9.78%, linoleic acid (C18:2) 9.56%, arachidic acid (C20:0) 0.36%, gondoic acid (C20:1)

0.39%, α-ESA 70.57% and β-ESA 3.25%. α-ESA was the principal fatty acid followed by oleic, linoleic, and β-ESA acids. These results are confirmed by the findings of John *et al*. (2002). The presence of high amounts of the essential conjugated fatty acids such as α-eleostearic acid have recently shown promise for anticancer applications and industrially as drying agents in paints, inks and varnishes (Koba *et al*., 2002 & Kohno *et al*., 2002).

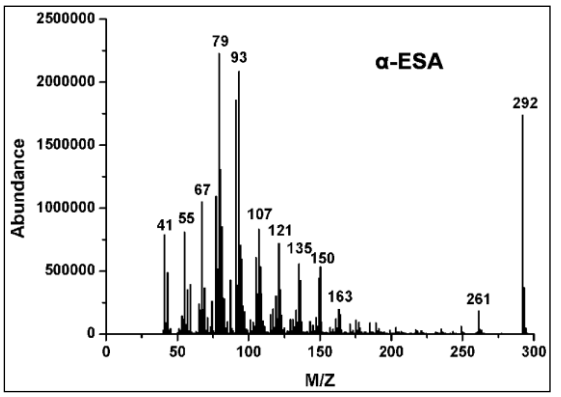

Figure 1. Mass spectra of α-Eleostearic acid methyl ester derived from Kan-zaw seeds

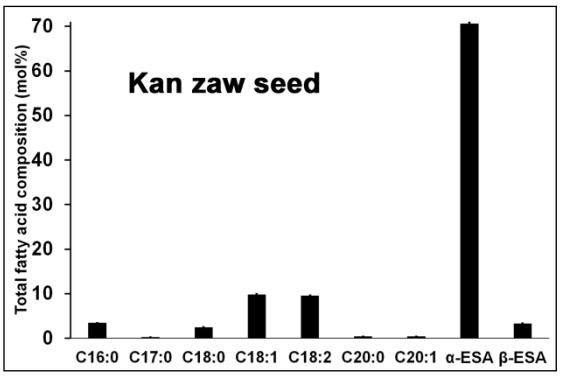

Figure 2. Functional analysis of labeled histogram correspond to the methyl esters of palmitic (16:0), stearic (18:0), oleic (18:1), and linoleic (18:2) acids. The GC chromatogram of FAME derived from (Kan-zaw oil) to illustrate the positions of α-Eleostearic and β-Eleostearic acid methyl esters

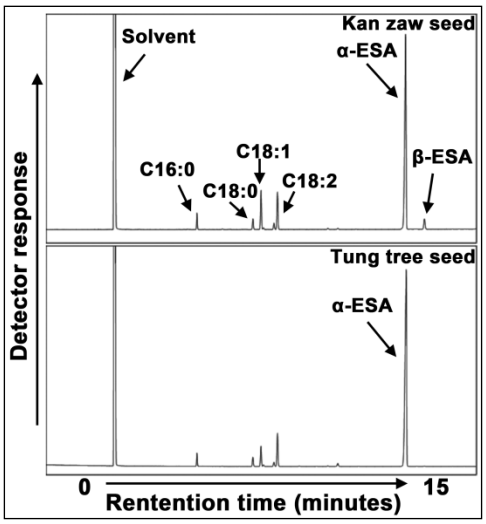

Figure 3. GC analyses of FAMEs extracted from Kan-zaw seeds. Standard includes FAMEs from Tung seeds (*Aleurites fordii* Hemsl.) and fatty acid α-ESA, and β-ESA, respectively from Kan-zaw seeds

Table 1. Fatty acids composition (%) of Kan-zaw seeds.

Fatty acids	**Determined value %**
Palmitic acid (C16:0)	3.41
Margaric acid (C17:0)	0.23
Stearic acid (C18:0)	2.45
Oleic acid (C18:1)	9.78
Linoleic acid (C18:2)	9.56
Arachidic acid (C20:0)	0.36
Gondoic acid (C20:1)	0.39
α - Eleostearic acid	70.57
β - Eleostearic acid	3.25

Anticancer activity by MTT assay

The results for cell growth inhibition by the extract against HeLa cell lines for various concentration are shown in Fig. 4, 5, 6 and Table 2, 3, 4, 5, 6. In the present study the percentage of viable cells remained more than 73% even when cells were treated with 100 µL of concentration for 24 h. But when the doses were increased, the percentages of viable cells were decreased and finally at a dose of 250 mg/l of concentration over 10% cells were viable. The extracts of ethanol, methanol, acetone, chloroform of Kan-zaw seeds and commercial Kan-zaw oil were effective towards tested cell line, among these commercial Kan-zaw oil was found that with the highest 88.32%. These results indicate that concentrations show significant potentiality against the viability and proliferation of cervical carcinoma cell (HeLa cell) line.

The acetone and ethanol extracts of leaves of *Madhuca longifolia* shows the cytotoxic activity against Ehrilich Ascites Carcinoma cell lines using different *in vitro* cytotoxicity assay at 200 g/ml (Yadav *et al*, 2012). The utility of cell lines acquired from tumors allows the investigation of tumor cells in a simplified and controlled environment (Arya *et al*., 2011). In the present study the HeLa cell lines are used as a model for studying cervical cancer. Several mechanisms of action were detected in HeLa cells. Patel *et al.* (2009) stated that the % growth inhibition increasing with increasing concentration steadily up to 0.0196-10 mg/ml *Solanum nigrum* effect on HeLa cell Line.

The IC50 of extract on cell line less than 100 g/ml is categorized as a potential cytotoxic substance (Spavieri *et al*., 2010). In the present study, ethanol, methanol, acetone, chloroform extracts and commercial Kan-zaw oil were found to be moderately cytotoxic towards human HeLa cell in MTT assay.

Table 2. For percentage (%) of cell growth inhibition of ethanol extract of *Payena paralleloneura* Kurz. (Kan-zaw) seeds on HeLa cells by MTT assay.

Concentration of the Extracts (mg/l)	**Absorbance**	**Inhibition of Cell Growth (%)**
250	0.076	87.14
200	0.391	33.84
150	0.415	29.78
125	0.434	26.57
Control	0.591	0

Table 3. For percentage (%) of cell growth inhibition of methanol extract of *Payena paralleloneura* Kurz. (Kan-zaw) seeds on HeLa cells by MTT assay.

Concentration of the Extracts (mg/l)	**Absorbance**	**Inhibition of Cell Growth (%)**
250	0.109	81.56
200	0.219	62.94
150	0.330	44.16
125	0.385	34.86
Control	0.591	0

Table 4. For percentage (%) of cell growth inhibition of acetone extract of *Payena paralleloneura* Kurz. (Kan-zaw) seeds on HeLa cells by MTT assay.

Concentration of the Extracts (mg/l)	**Absorbance**	**Inhibition of Cell Growth (%)**
250	0.074	87.48
200	0.081	86.29
150	0.437	26.06
125	0.447	24.37
Control	0.591	0

Table 5. For percentage (%) of cell growth inhibition of chloroform extract of *Payena paralleloneura* Kurz. (Kan-zaw) seeds on HeLa cells by MTT assay.

Concentration of the Extracts (mg/l)	**Absorbance**	**Inhibition of Cell Growth (%)**
250	0.071	87.99
200	0.170	71.24
150	0.282	52.28
125	0.289	51.1
Control	0.591	0

Table 6. For percentage (%) of cell growth inhibition of commercial oil of *Payena paralleloneura* Kurz. (Kan-zaw) seeds on HeLa cells by MTT assay.

Concentration of the Extracts (mg/l)	Absorbance	Inhibition of Cell Growth (%)
250	0.069	88.32
200	0.322	45.52
150	0.366	38.07
125	0.379	35.87
Control	0.591	0

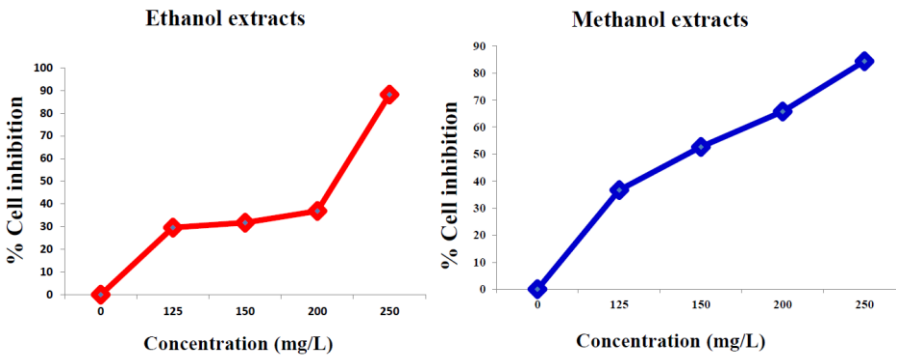

Figure 4. Effect of ethanol and methanol extracts of Kan-zaw seeds on HeLa cells by MTT Assay

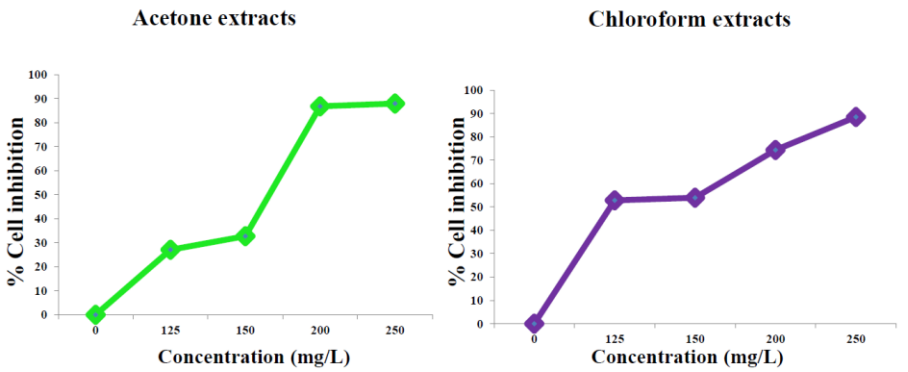

Figure 5. Effect of acetone and chloroform extracts of Kan-zaw seeds on HeLa cells by MTT Assay

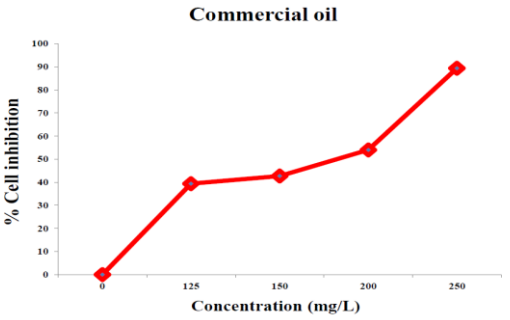

Figure 6. Effect of commercial oil of Kan-zaw seeds on HeLa cells by MTT Assay

Conclusion

The seed oil derived from the Kan-zaw owing to their excellent conjugated fatty acids approximately >70% α-eleostearic acid and β-eleostearic acid methyl esters are naturally occurring compounds that have specialized uses in nutraceutical (anticancer) and industrial applications. The Kan-zaw oil is famous in Myanmar. Although the oil is popularly used by local people and have commercial value, the scientifically research of this plant has not been undertaken previously.

Various extracts of Kan-zaw seeds were prepared for the first time to the best of my knowledge and the synthesized concentration showed a

significant efficacy against cervical carcinoma (HeLa) cell line with different concentrations along with >80% cell killing potentiality. Now overall study evaluate that *Payena paralleloneura* Kurz. (Kan-zaw) various extract and commercial oil exhibit potential effects against (HeLa) cell line in a dose dependent manner. On the basis of review of literature it concluded that research performed on seeds of Kan-zaw to highlight its medicinal properties, but only few experimental research have not been performed for utilizing it as a medicine.

The anticancer property of *Payena paralleloneura* (Kan-zaw) will provide a useful information in the possible application in the prevention and treatment of cancer. So now the time of diversion for commercial utilization of Kan-zaw seeds will also be used in preparation of medicines. This effort may increase the employment and income generation potential of the nation. Further research based on animal models will be conducted *in vivo* efficacy of Kan-zaw seeds.

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