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Research article

PHYTOCHEMICAL ANALYSIS, TOXICITY AND CYTOTOXICITY EVALUATION OF *DENDROPTHOE PENTANDRA* LEAVES EXTRACTS

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ABSTRACT: *Dendrophthoe pentandra* known as mistletoe is a semi-parasitic plant with traditional claims for some medicinal properties. This research was done to determine phytochemical constituents of *Dendropthoe petandra* (DP) leaves extract, to evaluate toxicity of extracts by brine shrimp lethality test (BSLT) and to confirm cytotoxicity activity of DPME against various normal cell lines. The most potent extract was then evaluated by GC-MS. DP leaves that have been extracted with petroleum ether (DPPEE), methanol (DPME) and water (DPWE) were screened for phytochemical constituents. BSLT was carried out to determine the lethality concentration that kills 50% of tested population (LC_{50}). The cytotoxicity was assessed by Methylene Blue Assay (MBA) that evaluates the inhibition concentration for cell growth by 50% (IC_{50}). The normal cell lines used were MDCK, L929 and Vero. Phytochemical analysis revealed the presence of alkaloid, flavonoid, saponin, tannin and terpenoid in those extracts. Highest total phenolic content was found in DPME (471.63±2.02 mg GAE/g). BSLT have determined the lowest LC_{50} value is 2.74±1.23 ppm in DPME. No IC_{50} detected when MDCK, L929 and Vero cell line were treated with all extracts. Therefore, this can be concluded that DP extracts did not show any harmful effects towards MDCK, L929 and Vero cell lines although the DPME, DPPEE and DPWE are toxic towards brine shrimp. Hexadecanoic acid, methyl ester and 9,12,15-Octadecatrienoic acid, methyl ester are among compounds present in DPME. Further studies using mammalian cancer cell lines should be conducted on DP extracts to know if they posses anticancer potential.

Key words: *Dendrophthoe pentandra*, Phytochemical, Toxicity, Brine Shrimp Lethality Test, Cytotoxicity, Gas Chromatography – Mass Spectrophotometry

INTRODUCTION

Dendrophthoe sp is one of genera in the Loranthaceae family. There are many species under the genera *Dendrophthoe*, and one of those species is *Dendrophthoe pentandra* (DP). It is widely distributed in China, Cambodia, India, Indonesia, Laos, Malaysia, Myanmar, Philippines, Thailand, and Vietnam (Qiu and Gilbert, 2003). DP known as mistletoe is a parasitic plant that obtains their nutrition by living on other plants. Traditionally, this plant is used in the treatment for cough, diabetes, hypertension, cancer, diuretic, smallpox, ulcer, skin infection and after childbirth treatment in Indonesia (Artanti et al., 2012). In Sulawesi Island, this plant is used as medicine to cure cancer (Ishizu et al., 2002). According to Artanti and co-workers (2012), DP leaves extract shows significant antioxidant and antidiabetic activity. DP extract has high antioxidant activity with extract exhibit free radical scavenging activity with IC_{50} less than 50 µg/ml (Uji et al., 2007). Quercetin from the flavonoid's group is one of the compounds that have been found in DP that have high antioxidant activity (Uji et al., 2007). In Indonesia, the leaves of DP has been reported used in the traditional medicine to treat wounds and skin infection; while whole part of the plant is used to cure hypertension and cough (Ishizu et al., 2002). The decoction of DP plant is commonly consumed by Indonesian but also among Malay community in the treatment of cancer. The safety of certain extract can be said as an important tool for preliminary assessment of the traditional medicine. The brine shrimp lethality test or else known as brine shrimp lethality bioassay is a simplest, reliable, less expensive and easily achievable method replacing cell lines bioassay in order to determine the toxicity of plants extracts by the estimation of their medium lethality concentration LC_{50} (Akpemi, 2012). It is based on the killing ability of test compounds on a simple zoological organism-brine shrimp, *Artemia salina*. This lethality test is often used for preliminary screening before cytotoxicity assay using cancer cell line was carried out (Artanti et al., 2012).

To confirm the presence of bioactive compounds and phytochemical constituent that can display valuable and wide range of biological activities, the usage of gas-chromatography mass spectrophotometry (GC-MS) is much relevant.

Chromatography plays a fundamental role as an analytical technique for quality control and standardization of phyto-therapeuticals (Andrew, 2007). In recent years, the GC-MS studies have been increasingly applied for the analysis of medicinal plants as this technique has proved to be a valuable method for non-polar components and volatile compounds analysis (Sermakkani and Thangapandian, 2012). Compounds with higher polarity could be detected earlier than less polar compound because it has different interaction with the immobile phase of GC-MS (Anisa et al., 2011). As far as our literature survey could ascertain, there is no scientifically evidence and no extensive research involving different type of DP extracts that evaluate its biological characterization, safety and efficacy have been reported. Thus, the objective of this study are to screen the phytochemical compounds of DP leaves extracts, to investigate toxicity of DP leaves extract by brine shrimp lethality test and also to further confirm cytotoxicity level of extracts by methylene blue assay towards mammalian normal cell lines.

MATERIALS AND METHOD

Sample collection

DP plants were collected from Pasir Puteh, Kelantan, Malaysia. The samples were authenticated by botanist of Herbarium Unit, School of Biological Sciences, Universiti Sains Malaysia, Malaysia.

Extraction process

The leaves were washed, dried and blended into powdered form. The petroleum ether extract (DPPEE) was prepared by soaking powdered samples in petroleum ether and was placed on a shaker overnight at room temperature. The extracts were filtered and concentrated using rotary evaporator (Rotavac Heidolph Laborate 4000 series), followed by evaporating in fume hood for several days. The dried residue was further extracted with methanol to give methanol extract (DPME). The residue was used for water extract (DPWE) by reflux method. The extracts were filtered and put into freezer (-20^{o}C) and freeze dryer (ILshin Lab Co. Ltd, model TF05505) until the extracts become powder form. The extracts were then stored at 4°C for further usage.

Phytochemical screening

All types of extract undergo qualitative phytochemical analysis. A qualitative phytochemical test to detect the presence of flavonoids, alkaloids, saponins, tannins and terpenoids were carried out using standard procedures (Siddiqui et al., 2009; Manjamalai et al., 2010; Khan et al., 2011; Harborne, 1973; Swan, 1979; Brunner, 1984). The total phenolics content was determined by modified Folin-Ciocalteu method (Wolfe et al., 2003; Jimoh et al.,2008). Total phenolic content was expressed as mg/g gallic acid equivalent (GAE) for every gram of sample (mg GAE/g).

Toxicity Assay by Brine Shrimp Lethality Test (BSLT)

The BSLT was performed as have been reported by (Daniel et al., 2012) with slight modification. This test was carried out to determine the lethality concentration which concentration that required to kill 50% of the brine shrimp population after certain specified duration. Hatching process was started with 0.25 g of brine shrimp eggs (nauplii) were hatched in a well aerated flask containing 250 ml 3.8% of seawater. A bright light source was left on and the hatching process was left at room temperature for 48 hour. Then, the bioassay was carried out as 0.2 g of DPWE, DPPEE and DPME were dissolved in 100 ml of distilled water to make 2000 ppm concentration of stock solution. Serial dilution was carried out by preparing the 1000 ppm, 500 ppm, 250 ppm, 125 ppm, 62.5 ppm, 31.2 ppm, 15.6 ppm and 7.8 ppm. After 48 hours of hatching the brine shrimp, 5 ml of each sample dilution and 5 ml of seawater containing 10 brine shrimp eggs is incubated in petri dish. The petri dishes were left in room temperature for 24 hour. After 24 hour, each of petri dishes was examined by using magnifying glass and average numbers of nauplii survived are counted. This experiment was carried out in triplicate.

Cytotoxicity Evaluation by Methylene Blue Assay

Normal cell lines used were Madin-Darby canine kidney (MDCK), mouse fibroblastic (L929), and African green monkey kidney (Vero). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) medium supplemented with 5% (v/v) foetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotics. The cells were cultured in a 5% CO_2 incubator at 37^{o}C in a humidified atmosphere. The culture was subcultured every two to three days as needed and routinely checked under an inverted microscope (Leica DMIM & DMLD) for any contamination. Non-malignant cells with 80-90% confluence were used for plating. The adherent cells were trypsinized to detach cells. 100 µl of cells (5×10^4 cells/ml) were seeded into each well of the 96-wells microtiter plates (Nunc-Immuno). Plates were maintained at 37^{o}C in a humid incubator with an air mixture containing 5% (v/v) CO_2 for 24 hours until 80-90% confluency. Then, old medium were discarded and 200 µl of new medium were added. Next, the cells were treated with 2 µl of series dilution of plant extracts (0.39 µg/ml – 99.0 µg/ml). The cells were also treated with 2 µl DMSO as control. The plate was returned to incubator for 72 hours. All treatments were done in triplicate. After 72 hours treatment, anti-proliferative activity of plant extracts were studied using methylene blue assay (Lin and Hwang., 1991; Tan et al., 2005; Zazali et al., 2013). 22.5 µl of 25% glutaraldehyde was added into each well to fix the viable cells to bottom of the well and run off on shaker for 15 minutes. Glutaraldehyde and old medium were removed and dead cells were washed away with 100 µl 0.15 M sodium chloride (NaCl) for three times. Subsequently, the viable cells were stained with 100 µl of 0.05% methylene blue dye and run off on shaker for 15 minutes.

The balance of methylene blue was then removed and rinse with 0.15 M NaCl for three times. 200 µl of 0.33 M HCl was added and placed on shaker for 15 minutes to get good colour elution. Optical density was read using ELISA reader at 660 nm. The intensity of wavelength absorb is proportional to the live cells present in the wells. The IC_{50} value was determined using graph plotting percentage of viable cells versus $\log_{10}$ concentration (µg/ml) of extract. IC_{50} value of the extract was determined from the plot of viable cells percentage and final concentration of the extract. Viable cells percentage was counted as :

IC_{50} determination = [Mean of optical density of treated cells x 100%

Mean of optical density of control cells]

Gas Chromatography- Mass Spectrometer (GC-MS) Analysis

10 µg of sample extract was dissolved in 2 ml methanol and put into GC-MS vial for GC-MS analysis. GC-MS analysis was carried out on a GC Agilent Technologies 6890 Network System and gas chromatography interfaced to a mass spectrometer Agilent Technologies 5973 inert Mass Selective Detector. These instrument was employed the following condition : Column HP-MS (30 x 0.25 mm ID x 0.25 film thickness), operating in electron impact mode at 70 Ev, helium (99.999%) was used as carrier gas at a constant flow of 1.2 ml/min and injection volume of 1 µl was employed (splitless mode:, injection temperature 280°C). The oven temperature was programmed from 70°C (isothermal for 2 minutes) with an increased of 20°C/min, to 280°C for 30 min. The scan rate was 2.42s per scan. The solvent delay was 2 min and the total running time was 42.5 min. For identification of components, the interpretation on mass spectrum GC-MS was conducted using database of National Institute Standard and Technologies (NIST) 2002 having more than 62 000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components in the NIST library. The name, molecular weight and molecular structure of the components of the test materials were ascertained.

Statistical analysis

All data were conducted in triplicates. The data were analyzed using Graph Pad Prism 5. The results were presented in mean ± standard error mean (SEM). Probability values ($P<0.05$) were considered statistically significant.

RESULTS AND DISCUSSION

Phytochemical Screening and Total Phenolics Content

Table 1 : Qualitative phytochemical screening of DPPEE, DPME and DPWE

Phytochemical	DPPEE	DPME	DPWE
Alkaloid	-	+	+
Flavonoid	+	+	+
Tannin	+	+	+
Saponin	+	+	+
Terpenoid	+	+	-

Table 1 and 2 showed the phytochemical analysis of DP from different extract based on qualitative basis. The present phytochemical investigation revealed the presence of flavonoid, saponin and tannin in all extracts, while terpenoids were only present in DPPEE and DPME and alkaloid were only present in DPME and DPWE. The presence of flavonoids in DP extract is in line with the previous study which state that flavonoids mainly present in DP extract (Uji et al., 2007). Flavonoid are potent water-soluble antioxidants and free radical scavengers, which prevent oxidative cell damage and it does contain strong anticancer activity (Okwu and Josiah, 2006). This is in agreement with the findings of Artanti and co-workers (2012), who reported the leaves extract of DP have high antioxidant activities. The presence of saponin justifies the use of the DP extracts to stop bleeding and in treating wounds which has the precipitating properties and coagulating properties on red blood cells (Okwu and Josiah, 2006). Apart from flavonoid and saponin, DP also contains tannin. Tannin have stringent properties, hasten the healing of wounds and inflamed mucous membranes. These perhaps, explain why DP is used in treating wounds and skin infection (Uji et al., 2007). Besides that, tannin is a phenolics compound, and plant phenolics are a major group of compounds that act as primary antioxidant or free radical scavengers (Siddiqui et al., 2009). Tannin is widely distributed through the plant kingdom, almost found in every plant part which consist anticancer properties by blocking the production of enzyme required for cancer cell line growth (Mohammed, 2006. Another constituent present in DPPEE and DPME was terpenoids. The terpenoids group show significant pharmacological activities, including anti-viral, anti-bacterial, anti-malarial, anti-inflammatory, anticancer and inhibition of cholesterol synthesis activities (Nassar et al., 2010). This finding justifies the use of *Dendrophthoe sp* plant in treatment of diseases, such as diabetes, cancer and hypertension (Dai and Mumper, 2010).

The total phenolics content was expressed using the milligram per gram of gallic acid equivalent of extract (mg GAE/g). The results from this study showed that DPME had higher phenolic compounds than the other extract and thus could be more effective as an antioxidant. Phenolic compounds are very important plant constituents because this compound exhibit antioxidant activity by inactivating lipid free radicals and prevent decomposition of hydoperoxides into free radicals (Jimoh et al.,2008). Their scavenging activity is due to their hydroxyl group. The inverse relationship between food-based plant intake and the risk of oxidative stress associated disease such as cardiovascular diseases and cancer has been partially due to phenolics (McLaughlin et al., 1998).

Table 2 : Total Phenolics Content of DPPEE, DPME and DPWE

Phytochemical	DPPEE	DPME	DPWE
Total Phenolic Content (mg GAE/g)	179.31±6.37	471.63±2.02	280.07±3.90

Toxicity Assay by Brine Shrimp Lethality Test

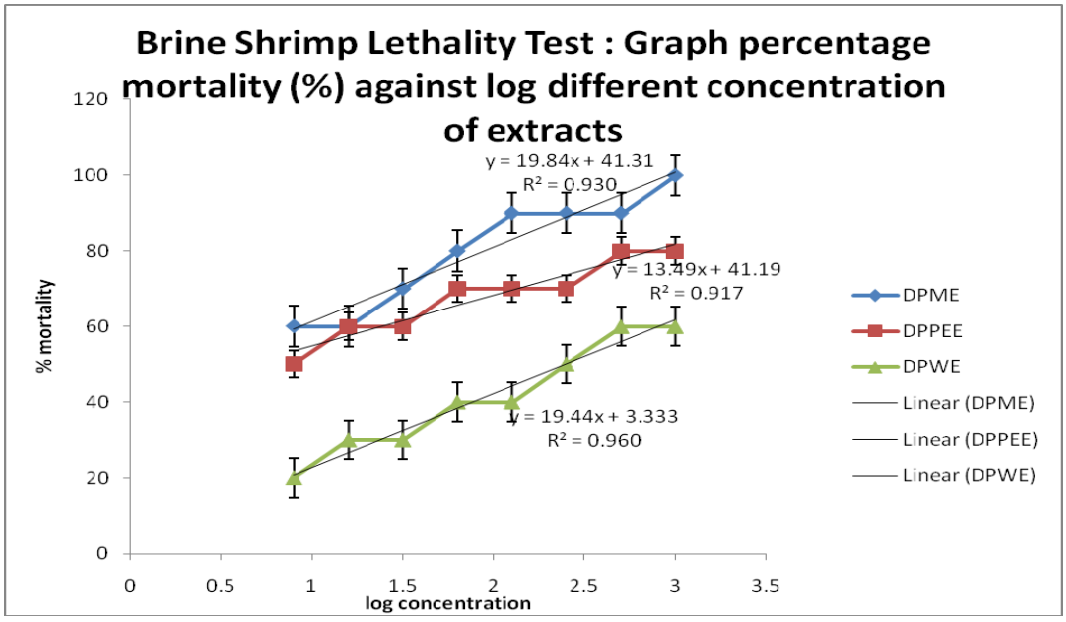

Figure 1 : A correlation between percentage mortality of brine shrimp and concentration (log) of DPME, DPPEE and DPWE.

Figure 1 showed the mean percentage mortality of the brine shrimp naupii against the logarithm of the DPME, DPPEE and DPWE several different concentrations. The mortality of brine shrimp increased with increasing concentration for both extracts. The LC_{50} value for DPME is 2.74±1.23 ppm, DPPEE is 4.50±0.87 ppm whereas that of DPWE is about 251.22±1.93 ppm. Standard brine shrimp lethality test stipulates that LC_{50} value less than 1000 ppm is considered bioactive in toxicity evaluation of plant extracts (Meyer et al., 1982). From this benchmark, the LC_{50} value for those extract implies that they are toxic. Comparison of the LC_{50} values of DPME, DPPEE and DPWE in Table 3 indicates that DPME is twice more potent than the DPPEE probably because it contains more active antitumor agents (Akpemi, 2012). To relate, the phytochemical test result shown in Table 1 and 2 clearly indicates that DPME contains more secondary metabolites with cytotoxic activity compared to DPWE. This accounts for why DPME's LC_{50} value is about twice more potent. The presence of saponins and terpenoid in extracts especially in DPME justified its potency as antitumor and anticancer agent (Mackeen et al., 2000) based on the LC_{50} values determined from brine shrimp lethality bioassay extracts or compounds that are cytotoxic have a good correlation to be effective antitumor agents (Man et al., 2010; Akpemi, 2012). This was in line with some other findings that reported that from pharmacological perspective, a good correlation has been found with brine shrimp lethality test to detect anticancer compound in plant extracts (Akpemi, 2012; Mackeen et al., 2000). By comparing the LC_{50} values of all extracts towards brine shrimp, it could be observed that DPME is more sensitive. This justified the efficiency of methanol as an extraction agent since methanol is more polar and lipophilic compared to ethanol.

Cytotoxicity activities by Methylene Blue Assay

Table 3 : Cytotoxicity activity of DPPEE, DPME, DPWE towards MDCK, L929 and Vero cell lines

Types of extracts/Cell lines used	IC50 value (µg/ml)		
	MDCK	L929	Vero
DPPEE	>100	>100	>100
DPME	>100	>100	>100
DPWE	>100	>100	>100

National Cancer Institute (NCI) has reported those crude extracts which demonstrate IC_{50} less than 20 µg/ml is considered to have good cytotoxic activity against cell lines (Faisal et al., 2012). To further confirm the cytotoxicity of those extracts, DPPEE, DPME and DPWE were screened by Methylene Blue Assay for cytotoxicity effect using normal mammalian cell lines such as MDCK, L929 and Vero cell lines. The representative results were shown on Table 3, Figure 2, Figure 3 and Figure 4. There were no IC_{50} detected in MDCK, L929 and Vero cell lines when tested with several concentrations of the extract as the IC_{50} value was more than 100 µg/ml. This indicates that the extracts does not show any cytotoxic effect on mammalian normal cells. No toxicity on this results of all extracts could be a good sign that this plant is relatively non-toxic, thus it is relatively save to be consume for traditional/alternative medicine.

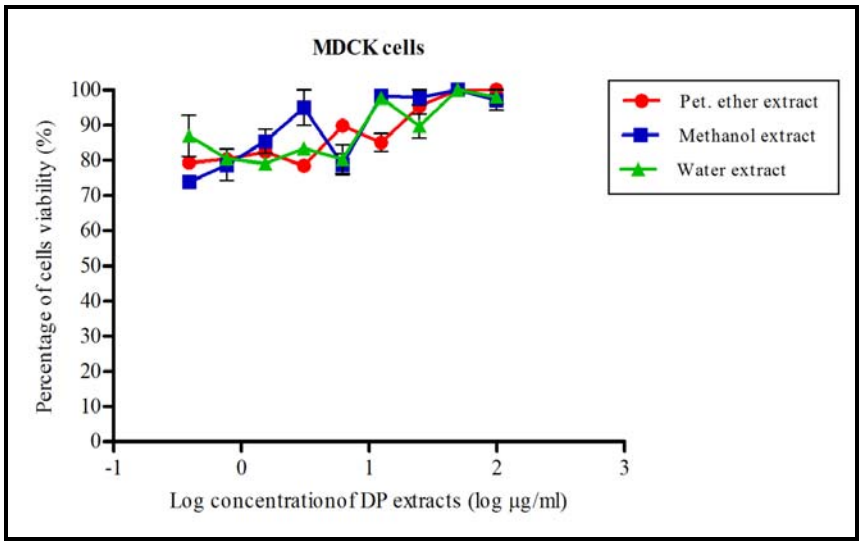

Figure 2: The percentage of cells viability of MDCK cells at different concentration of DPPEE, DPME and DPWE

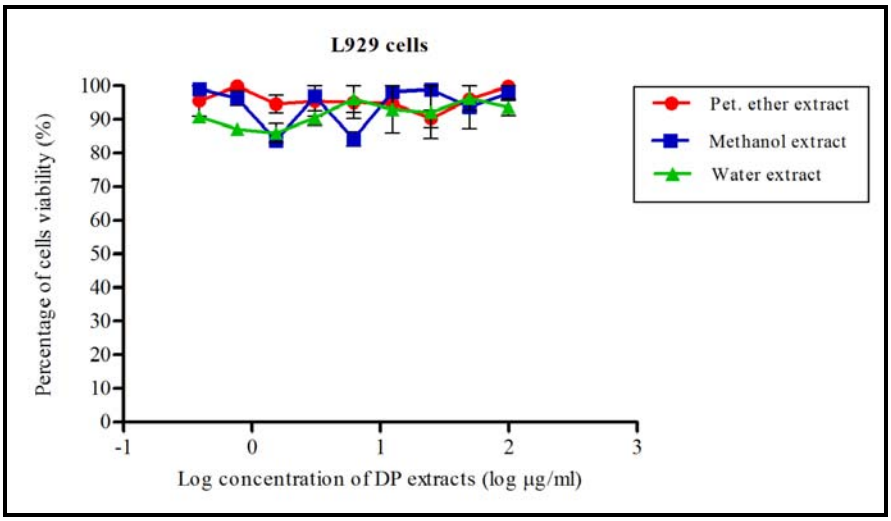

Figure 3: The percentage of cells viability of L929 cells at different concentration of DPPEE, DPME and DPWE

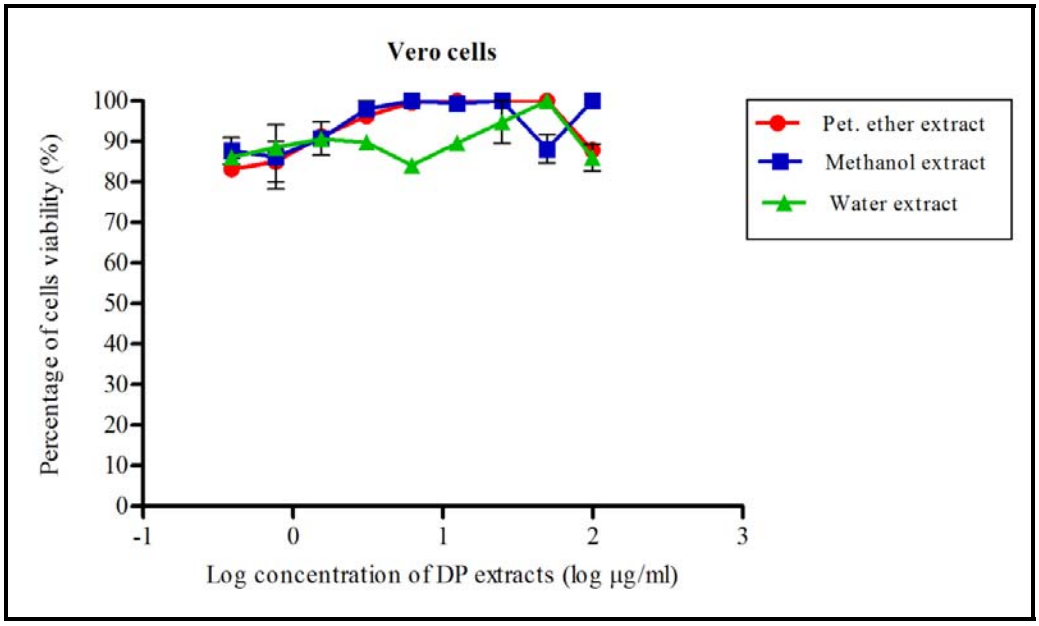

Figure 4: The percentage of cells viability of Vero cells at different concentration of DPPEE, DPME and DPWE

Since DPME showed most dominant extract in above biological assay, DPME have been evaluated through GC-MS to characterize the phytochemical compounds that been present earlier. This is the best techniques to identify the constituents of volatile matter, long chain, branched chain hydrocarbons, alcohols acids and ester (Sermakkani and Thangapandian, 2012). The identification of the compound was confirmed based on the peak area, retention time, molecular formula. The active principles with their retention time, name of the compound, molecular formula, molecular weight, peak area in percentage, compound nature and biological activity are presented in Table 4 and Table 5. The GC-MS analysis of DPME revealed the presence of five compounds (phytochemical constituents) that could contribute the medicinal quality of the DP leaves. The compounds were Benzoic acid, methyl ester, 2-Nonen-1-ol, Hexadecanoic acid, methyl ester, 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- and Nonadecanoic acid, methyl ester. For instance, 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- possesss anti-inflammatory, insectifuge, hypocholesterolemic, cancer preventive, nematicide, hepatoprotective, antiarthritic, anticoronary, antieczemic, antiacne, antiandrogenic and 5-alpha reductase inhibitor (Sermakkani and Thangapandian, 2012). This 9,12,15-Octadecatrienoic acid, methyl ester compound belongs to linoleic acid which has an inhibitory effect on human breast cancer cell lines which can be due to its two double – bondings molecular structure (Hasanzadeh et al., 2011). All of the bioactive compound that stated here are previously been reported from various number of other plant species.

Gas Chromatography- Mass Spectrometer (GC-MS) Analysis

Table 4: Bioactive components in DPME by GC-MS

No	RT	Name of compound	Molecular formula	Molecular weight	Peak area (%)
1	5.86	Benzoic acid, methyl ester	$C_8H_8O_2$	136.15	37.90
2	10.21	2-Nonen-1-ol	$C_9H_{18}O$	142.24	4.68
3	10.67	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.45	44.61
4	11.55	9,12,15-Octadecatrienoic acid, methyl ester	$C_{19}H_{32}O_2$	292.46	8.34
5	11.62	Nonadecanoic acid, methyl ester	$C_{20}H_{40}O_2$	312.53	4.46

Table 5 : The biological activities involved in DPME

Name of compound	Peak area (%)	Compound nature	Biological Activities
Benzoic acid, methyl ester	37.90	Fatty acid ester	• Antibacterial (Friedman et al., 2003)
2-Nonen-1-ol	4.68	Alcohol	• Flavouring agent (Reineccius, 1999)
Hexadecanoic acid, methyl ester	44.61	Fatty acid ester	• Anticancer, Flavour, Hypocholesterolemic (Sermakkani and Thangapandian, 2012). • Antioxidant (Kumar et al., 2010) • 5-alpha reductase inhibitor (Bhaskar and Selvamangai, 2012)
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	8.34	Linolenic acid	• Cancer preventive, Antiinflammatory, Hypocholesterolemic, Hepatoprotective, Nematicide, Antiarthritic, Anticoronary, Antiacne (Sermakkani and Thangapandian, 2012). • Antioxidant (Kumar et al., 2010)
Nonadecanoic acid, methyl ester	4.46	Fatty acid ester	• Antitumor, antioxidant (Gao et al., 2012)

Concisely, DP extracts did not show any harmful effects towards MDCK, L929 and Vero cell lines. The extract is found to be toxic in BSLT but not to be toxic in non-malignant cell line assay. It could be a good indication that the cytotoxic compounds in that extracts might have less toxicity and exhibit cytoselectivity towards mammalian normal cells since all treatment gave IC_{50} value more than 100 ug/ml. Although the DPME, DPPEE and DPWE are toxic towards brine shrimp, that results could be encouraging as evidence for the use of DP for cancer traditional/alternative medicine since BSLT is usually used for preliminary screening for bioactivity including for anticancer activities and compounds such as Hexadecanoic acid, methyl ester and 9,12,15-Octadecatrienoic acid, methyl ester that present in DPME are been previously reported effective in cancer inhibition.

CONCLUSION

By this, DPME is toxic than DPPEE and DPWE. The active compound that present in DPME should be isolated in order to determine biological active compound in the extract. Further studies using mammalian cancer cell lines could be conducted on DPME to determine its antiproliferative and anticancer potential.

CONFLICT OF INTEREST

We declare that we have no conflict of interest

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