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Pharmacological and toxicological activities of the extracts of papaya leaves used traditionally for the treatment of diarrhea

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ABSTRACT

The purpose of this study is to prepare various extracts with different polarity of solvent from the leaves which was collected from Malaysia and determine their antioxidant and cytotoxic activities by usual bioassays. The extract underwent successive fractionation and evaporation process by using different polarity of solvents to form corresponding hexane, ethyl acetate, dichloromethane, butanol extracts. All polarity extracts including water and methanol extracts were used for the determination of antioxidant and cytotoxic activities by using DPPH and BSL bioassays. The results of antioxidant activity showed that all the extracts from papaya leaves did not give much significant activity. However, among the extracts, the highest antioxidant activity was obtained in hexane extract and the lowest was in methanol extract. The range of IC_{50} values among the leaves extracts was 156.39–782.06 μg/ml and the height IC_{50} values was found in the hexane extract followed by hexane > dichloromethane > methanol > ethyl acetate > butanol > water extract. The cytotoxic results of all papaya leaves extracts showed significant activity against BSL bioassay. The highest cytotoxicity among them was in dichloromethane and the lowest was in water extract. In addition, the LC_{50} value among the different polarities leaves extracts, methanol extract showed significant cytotoxic activity having LC_{50} value of 118.73 μg/ml comparable to the other papaya extracts followed by dichloromethane > butanol > hexane > ethyl acetate > water extract. In conclusion, the results obtained from the present experiment, clearly showed that the nonpolar extracts are the best extract and it could be used as traditional medicine, food supplements as well as chemotherapy drugs.

1. Introduction

The application of plants and its processed products as herbal medicine has been used to treat various diseases since old times. Egyptians have traditionally used their locally available plants and its crude drugs for thousand years ago. Mesopotamia, people used some plants as formulated capsule and tablets as drugs to treat diseases and this are well documented in the pharmacopeia since the old times. The people of Mesopotamia still use most of the formulated drugs and plants for the treatment human diseases (Yadav et al., 2014). Taxol, vincristine and vinblastine are plant constituents which are widely used as chemotherapy drugs for the treatment of cancer. However, their side effect and toxicity against the normal cell which is associated directly with the applied dose are also reported. In this regards, alternative drugs or therapies against the cancer cell with less or without side effect on normal cells are highly required. Recently, several approaches *in vitro/vivo* anticancer screening have been used to estimate or evaluate the herbal drugs as well as crude extracts for their pharmacological, biological and toxicological profiling against the cancer cells as well as normal cells.

Plants as well as marine natural products are the vital source of herbal and pharmaceutical formulated drugs. It includes biologically active compounds from natural form that are difficult to isolate as well as expensive compared to synthesized pharmaceutical formulations one. As an isolated compound, the active compounds from nature which have been modulated is able to reduce toxicity, as well as improve the efficacy (Umamaheswari & Chatterjee, 2008; Jayaprakash & Rao, 2000). In addition, the plant active

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compounds are directly contributing to the discovery of pharmaceutical drugs. In this regards, it is important to screen the biological/ toxicological activities and phytochemicals of the traditionally used herbal medicines as well as the plant drugs for the treatment of human chronic diseases (Rani et al., 2016; Jain et al., 2011).

Papaya is a commoner and a delicious, economical fruit. It is scientifically known as Carica papaya which belongs to the Caricaceae family. Papaya originates from southern Mexico as well as Costa Rica. Today, papaya is grown in most countries including Oman. It also grows well in tropical and subtropical countries. It is a species of the flowering plants that is native to India, Malaysia, Indonesia, Philippines, Sri Lanka including Oman. Several Asian countries have cultivated the papaya on commercial basis. In some tropical countries, papaya is also cultivated as garden plant. The plant is a medium and a thinly branched tree with a single stem. The average height is about 5 to 10 m. The leaves of the plant are spirally arranged up to the top stem. Normally, the leaves are big with oval shape with about 20–28 in. diameter (Fig. 1). All parts of the plant contain white latex. The flowers are 5-parted pale white color petals and highly dimorphic. Both the male and female flowers are fused to the petals. The female flowers contain ovary and its five petals twisted loosely connected at the base (Wagner et al., 2012; Rossetto et al., 2008). The flowers are borne in the leaf axils. The fruits have different size and shape of about 5.9–17.7 in. long and 3.9–11.8 in. in diameter (Romasi et al., 2011). The ripen papaya fruits are soft and its skin turns into amber to orange color. Most parts of the plant like fruits, leaves, and flowers are edible. The plant has several chemical constituents such as polyphenols, glycoside, tannins, alkaloids, steroids, and saponins etc. The chemical ingredients catechuic acid, coumaric acid, methoxycoumarin, caffeic acid, kaempferol, quercetin, chlorogenic acid are present in the selected plant in high concentration (Ayoola and Adeyeye, 2010). The plant species are a powerful source antioxidant such as vitamin C, vitamin A and vitamin E. In addition, the plant also contains magnesium, potassium, B vitamin pantothenic acid and fiber (Wall, 2006).

All parts of papaya have medicinal values and have been used traditionally for the treatment number of diseases globally. Traditionally, it is used mainly to treat several conditions such as stomach disorders, diarrhea, skin diseases, male contraceptives, and home remedies for colds (Wall, 2006). Good numbers of studies have indicated that papaya possesses significant anticancer activities for colorectal, prostate, cervical and breast cancers (Aravind et al., 2013; Lohsoonthorn & Danvivat, 1995; Shahar et al., 2011; Pandey et al., 2017; Siegel et al., 2010). The extracts from the fruit, seeds, and leaves of the selected plant have also been shown to have significant cytotoxic activities against cancer cell lines including breast, liver and cancer of haematopoietic cell lines (Zhang et al., 2009; Nakamura et al., 2007; Garcia-Solis et al., 2009; Otsuki et al., 2010; Sancho et al., 2014). In Malaysia and Oman, papaya is used traditionally to treat diarrhea as well as gastric problems (Madhu et al., 2016). However, the phytochemicals and pharmacological studies have not been done extensively on this selected indigenous plant species. Therefore, the intention of the present study is to evaluate extensively the antioxidant and cytotoxic activities of the selected papaya species which is collected from Malaysia.

2. Materials and methods

In this present experiment, all chemicals and reagents were used at analytical grade. The acetone, hexane, dichloromethane, butanol, and ethyl acetate were purchased from different establish European Based Company. The purities of the solvents were about 98 to 99%. Shrimp eggs were bought from USA. Sodium chloride was purchased from the local market. DPPH (1,1-diphenyl-2-picrylhydrazyl) as well as DMSO solvent were obtained from the Sigma Aldrich Company, Germany.

2.1. Instruments

The absorbance of the prepared each concentration extract for the calculation of percentage of inhibition was measured by Shimadzu UV–visible spectrophotometer, Japan

2.2. Sample collection and identification

The leave samples were collected from the papaya producing farmer in Malacca, Malaysia. The samples were collected during the month of September 2018. Soon after collection, the leave samples packed in a plastic bag for travel and brought to the University of Nizwa Research Lab. Only the health papaya leaves were used for processing. The necessary process of the collected samples was done in the research lab 15L for extraction. The morphological features or identification of the leaves was done through website as well as the local people (https://en.wikipedia.org/wiki/Papaya).

2.3. Sample preparation

At first, the papaya leave samples were properly clean with water to eliminate filthy materials and kept at room temperature on newspaper until dried to obtain constant weight. The dried samples were crushed by using blender and made into a coarse powder. The course powder samples were kept at 4 °C in a safe place to avoid microbial and unintentional contamination.

Fig. 1. Picture of 1eaves of papaya.

2.4. Preparation of extracts and fractionation

The dried coarse powder (100 gm) was soaked with methanol (2 L) at room temperature for 48 h. During the extraction process the plant materials were stirred up and down using a glass rod for complete extraction. After 48 h of soaking, it was filtered by Buchner funnel to remove the unwanted coarse powder materials. Then, the filtrate was evaporated to dryness by using the usual rotary evaporator which gave an amorphous solid methanol extract (8.78 gm). From the methanol extract, approximately 7 gm was dissolved in water and transferred to the separatory funnel for fractionation (Weli et al., 2018). Various polarities of solvents such as hexane, dichloromethane, ethyl acetate, and butanol were used for successive fractionation to give hexane, dichloromethane, ethyl acetate, and butanol extracts which were evaporated to give the corresponding extracts. The water fraction was also evaporated and it considered as water extract. All the extracts were kept at 4 °C in a close container to avoid contamination as well as for further process.

2.5. Free radical scavenging bioassay

The free radical activity of the above various polarities extracts of papaya leaves was measured through 2,2-diphenyl-1-picryl-hydrazyl (DPPH) free radical bioassay. DPPH bioassay was performed according to the procedure described by Al Amri and Hossain (2018). The DPPH solution was prepared by dissolving 3.2 mg DPPH in 100 ml of analytical grade methanol. Approximately 2.7 ml of DPPH solution was added to each tube followed by the addition of 300 µl of a different polarity mother extract of each concentration. The final concentration was about 400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml, and 25 µg/ml. The final mixtures were shaken well by sonicator and kept in dark for incubation at room temperature for one and half hours. The absorbance of the incubated each concentration sample was measured by Shimadzu UV–visible spectrophotometer at the wavelength of 517 nm. The methanol was used as a blank while mixture of 300 µl of methanol and 2.7 ml of DPPH solutions were used as a control. Each concentration of the test samples was performed in triplicates. Finally, the percentage inhibition of each extract of various concentrations was measured according to the formula given below. The IC_{50} values of each extract were calculated by using graphical methods.

$$\text{Scavenging activity}\,(\%) = [(\text{Ac} - \text{As})/\text{Ac}] \times 100$$

where

Ac = means absorbance of DPPH
As = means absorbance of the test sample.

2.6. Cytotoxic bioassay

The cytotoxic activity of the above various polarities prepared extracts of papaya leaves was determined through brine shrimp lethality (BSL) bioassay. BSL bioassay was performed according to the procedure described by Al Alawi et al. (2018). In this present experiment, the artificial sea water was prepared by dissolving 38.2 mg of sodium chloride in 1000 ml of water for the hatching of the shrimp eggs. The artificial sea water solution (250 ml) was used in a duo plastic chamber and the shrimp eggs were added into the duo chamber and left for 24 h under the 60-watt light bulb. After 24 h incubation, the eggs were hatched and the larvae entered into another chamber. Various concentrations of each extract were prepared by using DMSO solvent. 4.9 ml of artificial sea water was added to the all working tubes followed by the addition of 100 µl each concentration of each extract. The final six concentrations were of 500 µg/ml, 250 µg/ml, 125 µg/ml, 65 µg/ml, 35 µg/ml and 15 µg/ml. The final mixtures were shaken well by sonicator and added with 10 larvae and kept in working tubes under the light at room temperature for 24 h incubation. The DMSO solvent was used as a control without extract while mixture of 4.9 ml of artificial sea water and 100 µl of DMSO solutions. Finally, the alive larvae were calculated by magnifying glass. The IC_{50} values of each extract were calculated by using graphical methods.

3. Results

The papaya leave samples were collected from Malaysia and the sample process and extraction were done at the University of Nizwa, Oman. The papaya leaves extract was prepared by soaking method with methanol. The methanol extract was dissolved in water and separated by various solvents with increasing polarity pattern. The percentage of the yield of methanol extract was 8.78%. However, after successive fractionation of mother extract, the highest amount was found was hexane and the lowest in ethyl

Table 1
Amount and percentage of each papaya leaves extracts.

Extracts	Amount (gm)	Percentage (%)
Hexane	2.98	42.57
Dichloromethane	1.36	19.43
Ethyl acetate	0.61	8.71
Butanol	0.89	12.71
Water	0.93	13.28
Methanol	8.78	8.78

Table 2
Percentage of free radical scavenging inhibition (%) of various papaya leaves extracts.

Extract	Concentration (µg/ml)	Absorbance (nm)	Free radical scavenging inhibition (%)	IC_{50} values (µg/ml)
Hexane	400	0.884	40.90	
	200	0.878	41.31	156.39
	100	0.772	48.39	
	50	0.771	48.40	
	25	0.442	70.45	
	400	0.927	38.03	
Dichloromethane	200	0.873	41.64	375.28
	100	0.846	43.00	
	50	0.743	50.33	
	25	0.774	48.26	
	400	1.132	37.00	
	200	0.944	41.64	422.24
Ethyl acetate	100	0.889	40.57	
Butanol	50	0.944	36.89	
	25	0.942	24.33	
	400	0.893	44.05	
	200	0.886	44. 38	
	100	0.903	40.77	703.12
Methanol	50	0.832	40.34	
	25	0.837	40.30	
	400	0.806	38.03	
	200	0.766	46.00	405.51
	100	0.877	48.79	
	50	0.872	41.71	
Water	25	0.966	35.00	
	400	0.810	45.83	
	200	0.862	42.37	
	100	0.840	43.83	782.06
	50	0.862	42.37	
	25	0.810	45.83	

acetate extract. The amount and percentage of each prepared extract is presented in Table 1.

3.1. Free radical scavenging activity

All the successive fraction extracts including methanol extract at various concentrations were used for the determination of free radical scavenging inhibition by using the modified DPPH method which was described by Al Amri and Hossain (2018). Most of the extracts from the papaya leaves did not showed promising activity against the free radical scavenging activity. The percentage inhibition was within the range of 35–70%. Among the extracts of papaya leaves, the most prominent activity was obtained from water extract and the lowest was in methanol extract. The free radical scavenging inhibition of each extract at various concentrations is presented in Table 2. The IC_{50} values of each extract of papaya leaves were calculated by using graphical methods. The highest IC_{50} values among the papaya leave extracts was obtained from water extract and the lowest was in the hexane extract (Table 2 and Fig. 2).

3.2. Cytotoxic activity

All the successive fractions of methanol extract as well as a mother methanol extract from the leaves of papaya at various concentrations were used for the determination of percentage of mortality by using the modified BSL method which was described in Al Alawi et al. (2018). Most of the extracts from the papaya leaves showed highest mortality against the BSL bioassay. Among them, the highest mortality was found in dichloromethane extract and the lowest mortality was obtained from water extract. The percentage of mortality of each extract at various concentrations is presented in Table 3. The LC_{50} values of each extract of papaya leaves were calculated by using graphical method (Fig. 3). The highest LC_{50} values were obtained

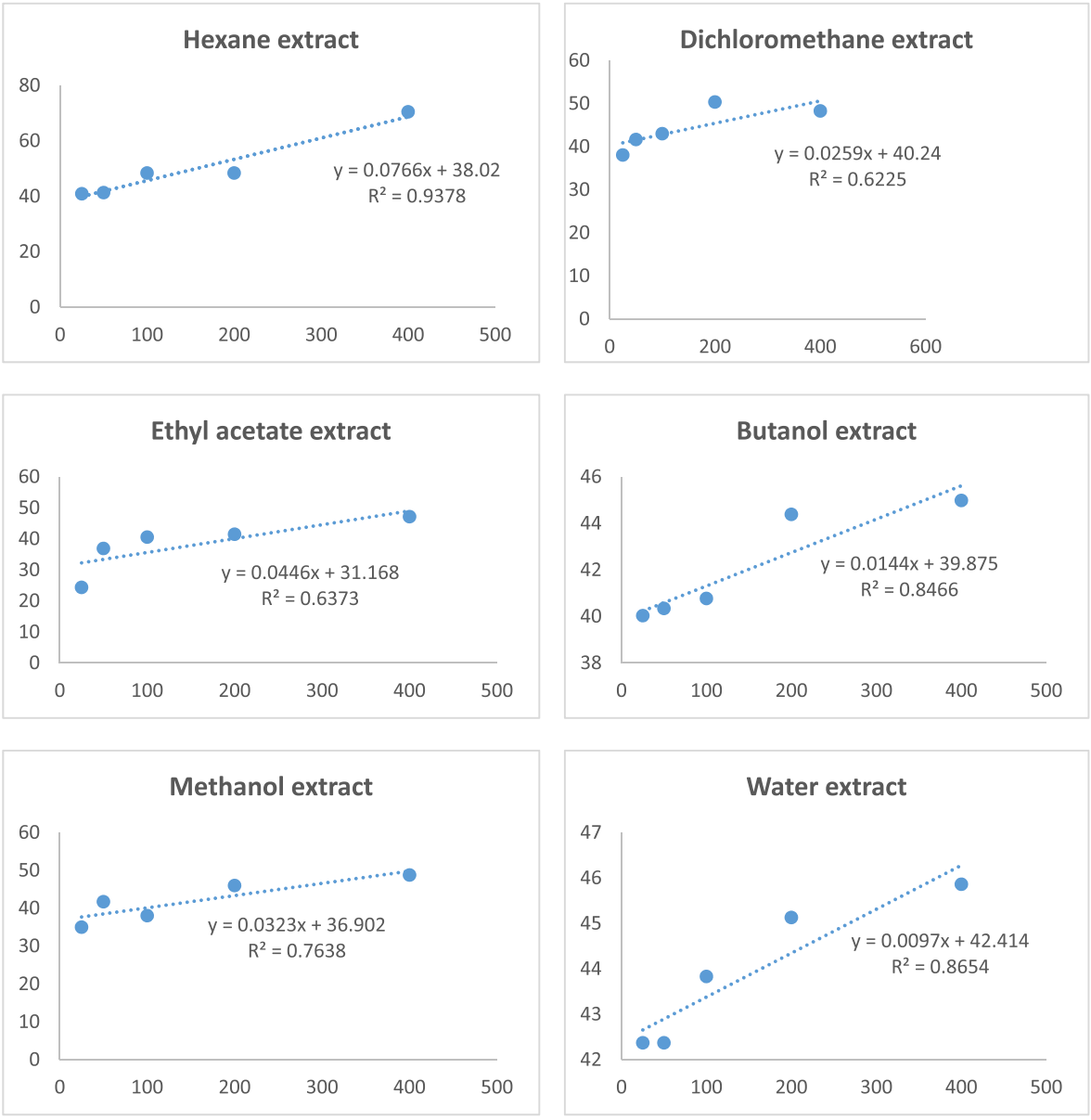

Fig. 2. IC_{50} values from scavenging inhibition (%) of various papaya leaves extracts.

Table 3
Percentage of mortality (%) of various papaya leaves extracts.

Extract	Concentration (ppm)	Alive (%)	Died (%)
Hexane	500	20	80
	250	50	50
	125	60	40
	65	70	30
	35	70	30
	15	80	20
Dichloromethane	500	30	90
	250	40	60
	125	40	60
	65	50	50
	35	60	40
	15	80	20
Ethyl Acetate	500	30	70
	250	40	60
	125	60	40
	65	70	30
	35	80	20
	15	90	10
Butanol	500	0	10
	250	30	70
	125	60	40
	65	70	30
	35	80	40
	15	80	40
Methanol	500	30	70
	250	30	70
	125	40	60
	65	50	50
	35	60	40
	15	70	30
Water	500	0	40
	250	0	20
	125	0	20
	65	0	10
	35	0	10
	15	0	10

from water extract and the lowest was in the methanol extract (Table 4 and Fig. 3).

4. Discussion

Free radicals defined as the chemical ingredients that are able to contain one or more unpaired electron outer shell. They are highly reactive as well as highly unstable (Kaur & Kapoor, 2002; Martınez-Cayuela, 1995; Schoneich, 1999). Some oxygenated species, especially superoxide radical (O_2^{-}), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$) are the most potent biological significance. Those are highly reactive and are potentially damaging the intermediate chemical species. On the other hand, cancer is the most common death causing disease globally, including the US (Atlanta, 2006). In the US, yearly about 1.6 million people were estimated to have been diagnosed with cancer. It is also estimated that approximately 0.7 million people are expected to die from various cancers in the coming years (Atlanta, 2006). According to the data reported by several authors, roughly 14 million cases of cancer reported globally and approximately half of them will be died from the cancer diseases (Siegel et al., 2017). National Cancer Institute US, recently published a report in 2016, that among the various type of cancers, breast cancer is identified as the most common followed by prostate cancer and lung cancer. However, statistic has indicated that lung cancer causes more deaths than breast or prostate cancer (Ferlay et al., 2015). According to the literature, breast cancer is phenotypes that based on three receptors: estrogen receptor, progesterone receptor as well as epidermal growth factor receptor (Ferlay et al., 2015; Al Alawi et al., 2018). Both the primary and advanced hormone breast cancers can be treated with endocrine therapy, which blocks both estrogen production as well as inhibits the estrogen receptor (Davidson et al., 2016). Recently, there has a been a development humanized monoclonal antibody which is approved by the Food and Drug Administration (FDA), USA for the treatment of breast cancer (Valabrega et al., 2007; Dean-Colomb & Esteva, 2008). In addition, the treatment of breast cancer is to grow inhibitors in the human body for angiogenesis. In this process, the new blood vessels are formed from the existing blood vessels (Jain & Carmeliet, 2001). On the other hand, the angiogenesis can be reduced drastically by several ways (1) to develop antibodies or small molecules against vascular endothelial growth factor which can inhibit the action of these proangiogenic factors and (2) to use of endogenous angiogenesis to inhibits the thrombopondin-1, endostatin, angiostatin, etc. (Shih & Lindley, 2006). All these latest process as well as technologies is successful, however, those kinds of treatments for cancer have tremendous side effects. Even though, on the patients besides developing drug resistance. Therefore, nowadays there is a growing interest to use natural products or drugs as an alternative or an adjunct strategy to treat as well as to prevent cancer including breast cancer.

4.1. Plant extract

The weight of various extracts obtained from the papaya leaves by using the soaking method and the percentage of yield is shown in Table 1. The weight of the methanol extract was an approximate 8.78 gm ≈ 8.78%. The methanol crude extract was fractioned by using various polarities of solvent starting from hexane. Among the fractionated extracts, the highest yield was obtained from hexane extract and the lowest yield was obtained from ethyl acetate which is presented in Table 1.

4.2. Free radical scavenging activity

The plant's medicinal value depends on the presence of chemicals that showed positive pharmacological and physiological actions on the human body which can be protected and treating human diseases. In the past studies on papaya plant showed that the selected plant contains secondary metabolites (Ayoola & Adeyeye, 2010). They are connected with the biological and pharmacological properties like antioxidant, antimicrobial, anti-inflammatory, antidiabetic etc. (Weli et al., 2018; Al Amri and Hossain, 2018). The previous studies also showed that the selected plant has several groups of chemical such as alkaloids, terpenoids, carbohydrates, tannins, flavonoids, saponins and steroids in various polarities of extracts in consideration amounts (Wall, 2006; Ayoola & Adeyeye, 2010). Nowadays, the antioxidants from natural sources are highly and well accepted over synthetic antioxidants due to their toxicity in the human body. There are several methods available to determine the antioxidant activity. Among them, the DPPH bioassay is the most popular and well accepted method for the determination of antioxidant activity of plant products. In our experiment, the scavenging activity of various papaya extracts was measured by using DPPH bioassay. In this method, DPPH is used to measure ability to donate the electron and radical scavenging ability of products (Al Amri and Hossain, 2018). The electron is transferred from antioxidant to DPPH radical molecules (Al Amri and Hossain, 2018). Therefore, the DPPH is acts as a reducing agent and thereafter DPPH lose its deep violet colour gradually. The change of colour was measured by using UV–visible spectrophotometer at the wavelength 517 nm. The antioxidant activity as a percentage of inhibition of various extracts of papaya leaves was calculated by using the well-established formula. The range of inhibition of various extracts of papaya leaves was from 35 to 70.35%. Among the six prepared extracts from the leaves, the

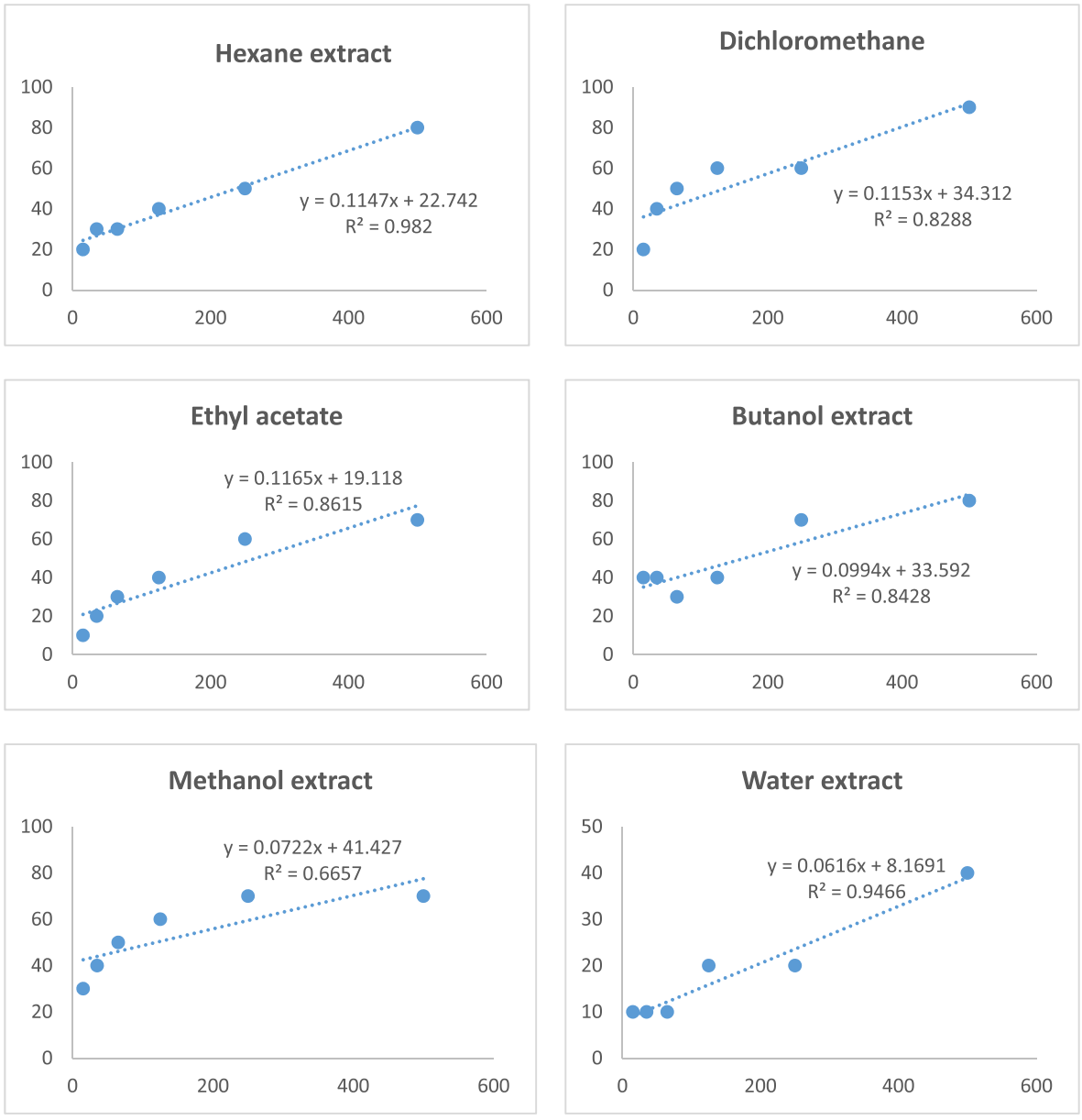

Fig. 3. LC_{50} values from percentage of mortality (%) of various papaya leaves extracts.

Table 4
LC_{50} values of various papaya leaves extracts.

Extracts	LC_{50} values (µg/ml)
Hexane	237.64
Dichloromethane	136.06
Ethyl acetate	265.08
Butanol	164.40
Water	679.07
Methanol	118.73

highest inhibition was in water extract and the lowest was in ethyl acetate extract followed by butanol > hexane > dichloromethane > methanol > ethyl acetate extract. The high activity in water extract may be due to the antioxidant guided most of the compounds present in this extract. In addition, the water is highly polar solvent and the water soluble compounds are also polar. Therefore, most of the antioxidant guided compounds are polar in nature. The LC_{50} of all prepared extracts of the papaya leaves was calculated in various concentrations of each extract against the percentage of inhibitions (Fig. 3 and Table 4). According to Table 4, the highest IC_{50} values were obtained from water extract (782.06 µg/ml) and the lowest was in the hexane extract (156.39 µg/ml) comparing to the original methanol extract and other extract of papaya leaves followed by water > butanol > ethyl acetate > methanol > dichloromethane > hexane extract. However, the toxicity is high in hexane and lower in water extract, followed by hexane > dichloromethane > methanol > ethyl acetate > butanol > water extract. Literatures from past studies have shown several previous reports on antioxidant activity of papaya leaves (Banu & Cathrine, 2018; Gotink and Verheul, 2010; Ang, 2012). However, our experimental results do not align with the reported data (Gotink and Verheul, 2010; Ang, 2012). The main reasons are i. our sample and extraction process were not similar ii. They prepared extract directly from the leaves while in this study fractionation process was used and iii. The presence of phytochemicals was in the extracts. Thus, these differences have made the results not aligned with the reported values.

4.3. Cytotoxic activity

The modification of the procedure described in Al Alawi et al. (2018), the lethality of various extracts of papaya leaves on brine shrimp was evaluated and the results of percentage mortality at various concentrations were shown in Table 3. Among the papaya leaves extracts, hexane, dichloromethane, ethyl acetate and methanol extracts showed promising mortality. However, the butanol and water extracts have lower mortality compared to other extracts. In addition, another indication is that the mortality of the extract is decreasing by the decreasing of concentration of each extract. It was noted that all the extracts from the leaves of papaya at 500 μg/ml produce significant percentage of mortality. The results showed that the highest mortality of about 90% was obtained from the dichloromethane extract at the concentration 500 μg/ml. However, the lowest mortality of about 40% was obtained from water extract. The dichloromethane showed that the highest mortality could be due the presence of highest number or concentration of toxic chemicals. In this result, it is clear that most of the toxic compounds are present in the dichloromethane extract and lesser number or concentration in water extract. Some correlation was observed when log concentration was plotted against the percentage of mortality (Al Alawi et al., 2018) on the graph paper. The LC_{50} values of each extract were calculated using Microsoft Excel 2010 (Fig. 3 and Table 4). According to the Table 4, methanol leaves extract showed significant cytotoxic activity having LC_{50} value of 118.73 μg/ml comparable to the other polarity papaya extracts. The dichloromethane and butanol also showed the potent LC_{50} activity. From the results, it indicates that most of the toxic compounds present in the methanol extract followed by dichloromethane > butanol > hexane > ethyl acetate > water extract. Several literatures on cytotoxic activity of papaya leaves have reported significant cytotoxic activity, however, this study does not align with the reported data (Nguyen et al., 2016; Shahar et al., 2011; Garcia-Solis et al., 2009; Otsuki et al., 2010). It could be due to the sample processing, extraction pattern from the plant samples, solvent–solvent fractionation as well as phytochemicals.

5. Conclusion

In conclusion, the biological and toxicological study of papaya leaves of various polarities extracts was done and the extracts showed promising activity when antioxidant and cytotoxic screened activities against DPPH and BSL bioassays. In our cytotoxic experimental, methanol extract showed the highest cytotoxicity among the six prepared extracts. Similarly, in antioxidant activity, the hexane extract showed the IC_{50} compared to other extracts. However, further studies are required to determine the actual chemical nature of these compounds and their mechanism of action.

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