Analysis of *Peganum harmala*, *Melia azedarach* and *Morus alba* extracts against six lethal human cancer cells and oxidative stress along with chemical characterization through advance Fourier Transform and Nuclear Magnetic Resonance spectroscopic methods towards green chemotherapeutic agents

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**Abstract**

Traditional medicines implicate consumption of plant crude extracts, which may consist of extensive phytochemical diversity. Overall, the most biologically active extract of *Peganum harmala* (seeds) exhibited significant cytotoxic activity on *Artemia salina* with LC$_{50}$ value of 61.547 mg/mL, while *P. harmala* (roots) [LC$_{50}$ = 124.229 mg/mL] and *M. azedarach* (fruits) [LC$_{50}$ = 147.813 mg/mL] showed moderate cytotoxic potential. *P. harmala* (seeds) extract also showed the maximum antitumor potential with 52.278 mg/mL LC$_{50}$. Branches of *P. harmala* and *M. alba* were not active in both bioassays. These outcomes were further reinforced by the levels of phenolics and flavonoids checked against gallic acid and quercetin equivalents, respectively, by standard curves. Current study aims to isolate, structurally characterize and analyze the bioactive compound from plant extracts by using chromatographic and spectrophotometric techniques. Bioactivity guided isolation of extracts led to the isolation of PH-HM-16 from ethyl acetate fraction of *P. harmala* seeds. Chemical structure of PH-HM-16 was elucidated by ESI-MS, $^1$H NMR, $^{13}$C NMR, HSQC and IR spectrum. The results demonstrated significant positive anticancer activities against six human cancer cell lines assessed through MTT cancer cell growth inhibition assay. PH-HM-16 was most effective against prostate cancer cell lines [IC$_{50}$ = 17.63 mg/mL] followed by breast cancer cell line MCF7 [IC$_{50}$ value of 41.83 µg/mL]. IC$_{50}$ value of PH-HM-16 against human myeloid leukemia cell line HL-60 and human colorectal tumor cells HCT-116 was observed as 68.77 µg/mL and 71.54 µg/mL respectively. The IC$_{50}$ value of PH-HM-16 compound was not significant against human gastric cancer SGC-7901 (111.89 µg/mL) and human lung adenocarcinoma epithelial cell line A549 (176.04 µg/mL). Isolated bioactive metabolite PH-HM-16 possesses significant antitumor potential so this could be the first step to develop an effective anticancer agent. Hence, this compound represents a promising potential to be chemically standardized or developed into pharmaceuticals for the chemoprevention and/or the treatment of certain types of cancer, especially as adjuvant phytotherapeutics in conventional chemotherapy.

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1. Introduction

Medicinal plants are an unlimited bioresources of therapeutic agents in conventional and traditional systems of medicine, food supplements, nutraceuticals, lead compounds or intermediates for synthetic drugs (Ncube et al., 2008). Traditional herbal remedies utilized for diverse bioactivities may work as a basis to
recognize novel compounds that can be candidates for the development of new anticancer drugs. In particular, it may be assumed that the cytotoxic action of plant extracts points to diverse healing applications. Plants with cytotoxic activity may be not merely active against tumors, but also as abortifacients, contraceptives and against infectious maladies caused by protozoans. In this sense, cytotoxic properties are often indicative of anticancer potential (Mariani et al., 2014; Monteleone et al., 2012; Leonti et al., 2015). Ethno-historic records indicate that natural medicinal products are powerful reservoirs of effective phytotherapeutics with rarer side effects (Umadevi et al., 2013; Srinivas and Afoyalan, 2007; Efferth et al., 2008).

*Melia azedarach* L. is prescribed in Chinese traditional medicine both orally and topically as antiparasitic and antifungal agent. This plant is a blood purifier and febrifuge whereas bark has vermicifuge action (Phua et al., 2008). Crushed leaves in water are effective in skin infections (Iqbal et al., 2011); leaf extract is a blood purifier and painkiller whereas dry foliage is used to cure jaundice, allergy, constipation and leprosy (Musthaq et al., 2012). Flowers and leaves are diuretic, emmenagogue and used to relieve headache and cold inflammation. Seeds are active in the treatment of rheumatism; seed powder is recommended for piles while seed oil is laxative, brain tonic and used in the treatment of earache and liver ailments (Ahmad et al., 2006; Iqbal et al., 2011). Roots are astringent, effective against vomiting, belching, heart disease, headache, fever, leukoderma, blood impurities, ulcer and uterine pains. Fruits are very useful as carminative and their powder are applicable for piles (Sultana et al., 2006).

*Peganum harmala* L. generally known as *harmala* is an extensively used medicinal plant of family Zygophyllaceae. Seeds are identified to be carminative, emetic, anthelmintic, galactagogue, aphrodisiac, diuretic, anti-inflammatory, emmenagogue, phlegmatic, purgative and strengthening the vision (Aghili, 2009; Tonkaboni and Momenin, 2007). They are used for psychosis, epilepsy, loss of memory, kidney stones, chronic headache, dropsy, rheumatism, colic, jaundice and sciatica (Aghili, 2009; Iqbal et al., 2011). Mixture of *P. harmala* and flax seeds along with honey has been used for the management of dyspnea. Decoction of seeds is efficient for treatment of laryngitis, sexual impotency, lung and liver diseases, as diuretic, demulcent and numbness (Ullah et al., 2013). Seed aqueous extract is beneficial for blood purification, while bark is well known for lowering blood pressure. Leaf extract is also widely utilized for wound healing, and sore throat as antiseptic and disinfectant (Gautam et al., 2007; Shinwari et al., 2003; Deshpande et al., 2008), as well as considered useful as anthelmintic, antibacterial, aphrodisiac, anti-inflammatory, diuretic, expectorant, antihypertensive, laxative and sedative agent (Dat et al., 2010; Husain et al., 2008; Khan et al., 2012a; Khan et al., 2012b; Qureshi et al., 2011).

Since ever, information on traditional herbal remedies has assisted in discovery of novel therapeutic leads with relevant bioactivity and less toxicity for cancer treatment, whereas basic research has emphasized their chemical modification and pharmacological profile (Bugs, 2004; Walsh and Amyes, 2004). This article aims to investigate the cytotoxicity and antitumor properties of different parts of important traditional plants for their development as possible source of phytotherapeutics as well as anticancer effect of isolated component against six selected human cancer cell lines.

2. Material and methodology

2.1. Plant selection

Three highly active species widely utilized as herbal remedies by local communities in salt range (Kallar Kahar, Pakistan) were selected for analysis (Table 1).

2.2. Plant extracts

*Melia azedarach* (leaves and fruits), *Peganum harmala* (roots, branches, leaves and seeds) and *Morus alba* (leaves and branches) were shade dried. The plant samples were extracted twice using methanol (95%) at 25 °C temperature for 7 days by using filter paper Whatman No. 1. Filtered samples were concentrated by rotary evaporation in reduced pressure at 40 °C and then stored at 4 °C for further in vitro investigations.

2.3. Cytotoxicity assays

2.3.1. Preparation of the bioassays

Bioassays were performed in multiwell plates (0.45 μm) and sterilized seawater. Extracts were tested at several concentrations achieved by shifting the corresponding stock volume to multiple wells for evaporation. Seawater (5 mL) was added to individual wells in aqueous solution and allowed to evaporate. All assays were conducted in a temperature-controlled room at 28 °C, under a constant light regimen.

2.3.2. BSLT (Brine Shrimp Lethality Test)

Dried cysts (1 g/L) were incubated in a hatcher with strong aeration at 28–30 °C. After hatching (about 12 h), the phototropic nauplii were picked from lighted section by a glass capillary tube and concentrated in a small vial. Every test consisted in exposing sets of ten Artemia (12 h aged) to several concentrations of extracts. Toxicity was evaluated after 12 h (generally nauplii in instar I/II) and 24 h (nauplii in instar II/III). Mortality percentage was calculated by noting dead and alive nauplii. The death of larvae observed in the bioassay is due to bioactives and not to starvation as by comparing dead larvae in treatment to control. Besides, hatched nauplii can stay alive up to 48 h even without food as they nourish on yolk sac. Nevertheless, if control mortality is noticed, mortality was estimated as:

\[
\text{Mortality} \% = \frac{\text{Survival in control (\%) - Survival in treatment (\%)}}{\text{Survival in control (\%) - Survival in treatment (\%)}}
\]

2.3.3. Toxicity testing criteria

Clarkson’s criterion for sample toxicity valuation categorizes in subsequent sequence: extracts with LC_{50} beyond 1000 μg/mL are...
considered as non-toxic; samples with LC50 in range of 500 to 1000 µg/mL are categorized as low toxic; extracts with 100–500 µg/mL LC50 are moderately toxic; whereas LC50 < 100 µg/mL are categorized as high toxic samples (Clarkson et al., 2004).

### 2.4. Inhibition of crown-gall tumorigenesis

*Agrobacterium tumefaciens* (At 10 strain) was grown at 28 °C for 48 h on yeast extract media. Segments of *Solanum tuberosum* L. were disinfected through immersing in mercuric chloride (10%) for half an hour. Cylinders (10 mm diameter) were made from the disinfected segments by sterilized cork borer; disks (0.5 cm diameter) were cut aseptically and placed in a 24 well culture plate comprising water agar (15%). Suspension of *A. tumefaciens* in phosphate buffered saline was standardized (1 × 10^9 CFU) through 0.97 ± 0.01 nm absorbance. Samples were prepared in Dimethyl sulfoxide (DMSO) in 1000 µg/mL and successively diluted to 500, 250, 125, 62.5, 31.25 and 15.625 µg/mL. Potassium dichromate, Vincristine and Etoposide were used as positive standards. Others constituents of bioactive component. The extract was adsorbed on a silica gel column by following the procedure of Hammad et al. (2015) with minor modification was used for purification of bioactive component. The extract was adsorbed on a silica gel column by using various mixtures of solvent system starting with non-polar and changed by slightly increasing polarity. A glass column will uniformly packed with slurry of silica gel in the mobile phase solvent system. The mobile phases were applied on the top of the column and continuous elution were collected. Ethyl acetate fraction was chromatographed over silica gel column packed in chloroform. Total of 30 elutions were collected using chloroform: methanol (1.0–0.1) as mobile phase with increasing volumes of methanol solvent. All the collected eluents were evaporated to dryness by removing mixture of solvents under reduced pressure through rotary evaporator at 40 °C to get dried concentrated eluents. Different components eluted through the column were collected in glass vials (20 mL) for further (Thin layer chromatography (TLC) analysis). Thus, the most bioactive material was purified using this silica gel column successively eluted with a stepwise gradient with increasing volume of solvent till total elution were collected. Aliquots of 15–20 µL of the isolated fractions collected by column chromatography were applied as separate spots on a TLC plate about 1.5 cm from the edge (spotting line). After sample application, the plate were placed vertically into a solvent vapor saturated TLC chamber (12 × 15 × 10 cm). After the mobile phase moved about 80% from the spotting line, the plate was removed from the developing chamber and air dried in a fume hood to visualize spots within 1–2 min on the plate. The migrated spots on TLC plates representing various fractions/compounds, visualized with UV lamp at UV-254 nm. All 30 elutions were subjected to thin layer chromatography with chloroform: methanol as solvent system with varying concentrations (Fig. 9: Supplementary data). On the basis of their TLC profile, the same elutions were pooled together to form Group 1 to Group 5. Elution showing similar pattern were pooled together and used for further analysis (Houghton et al., 1996). Since Group 2 showed significant activity compared to other groups, it was again subjected to silica gel column chromatography and 19 eluents were collected in vials (Fig. 9: Supplementary data). Solvents system used for this was n-hexane and methanol with gradual increase in methanol concentration (1.0–0.1). These eluents were again subjected to pre coated silica gel TLC plates and the same eluents were combined to form Group 2a (elution 1–6), Group 2b (elution 7–11) and Group 2c

### Table 1

| Plant Species | Family   | Local Name      | Part Used          | Traditional uses                                                                 | References                      |
|---------------|----------|-----------------|--------------------|----------------------------------------------------------------------------------|---------------------------------|
| *Morus alba*  | Moraceae | Toot, shahtoot  | Leaves, branches   | Pimples and warts, edema, eczema, swellings, antistress, inflamed eyes, astringent | Khan et al., 2012, Ahmad et al., 2007, Niyomkam et al., 2010, Leporatti and Gherdira, 2009, Dat et al., 2013, Naz et al., 2014, Iqbal et al., 2013 |
| *Melia azedarach* | Meliaceae | Dhraik          | Leaves, fruits     | Piles, skin diseases, blood purifier, anti-allergic, insecticidal, chicken pox, smallpox, tumors, inflammatory diseases, pimples | Iqbal et al., 2011, Naz et al., 2014, Faraga et al., 2013, Azam et al., 2013, Habir et al., 2015, Akhisa et al., 2013 |
| *Peganum harmala* | Zygophyllaceae | Harmal        | Roots, branches, leaves, seeds | Blood purifier, antiseptic, healing ulcers, skin diseases, pimples and lumps on skin, anti-inflammatory, insecticidal | Iqbal et al., 2011, Naz et al., 2014 |
(elution 12–19). HPLC analysis was carried out with photodiode array detector and LC column (150 × 4.6 mm). Two mobile phases A consisted of n-hexane and B ethyl acetate at flow rate of 0.5 mL/min were used. A gradient starting at 95% A changing to 10% was used for 10 min. After eluting, the column was equilibrated under initial conditions for 5 min. Total of 63 elutions were collected which were rotary evaporated and subjected to TLC. On the basis of TLC fingerprint profiling, similar groups were pooled together to get Group 2b.A, Group 2b.B, Group 2b.C and Group 2b.D. The isolated components were further characterized with the help of available spectroscopic techniques including Infrared spectroscopy (IR), Mass spectrometry (MS) and Nuclear Magnetic Resonance spectroscopy (NMR). Mass spectra of isolated components was obtained using mass spectrophotometer for molecular mass determination and ESI-MS spectral analysis was recorded in the positive ionization mode on mass spectrophotometer. IR Spectroscopy was measure the vibrations of atoms on the IR spectrophotometer (BUCK SCIENTIFIC) and based on this it was help to determine the functional groups. Nuclear Magnetic Resonance Spectroscopy (NMR) was performed with appropriate solvent to elucidate the structures of the isolated components (Vollhardt et al., 2007). Proton Nuclear Magnetic Resonance (1H NMR) Carbon Nuclear Magnetic Resonance (13C NMR) and Heteronuclear Single Quantum Coherence (HSQC) experiments were obtained on spectrometer (Bruker, 300 MHz, 500 MHz).

2.8. MTT cytotoxic assay

The growth of human cancer cell lines was evaluated using the colorimetric (3-(4,5-dimethylthiazol-2-yl)phenyl tetrazolium bromide) MTT assay (Hayoto et al., 2006). Cell lines were incubated in 96-microwell plates and the metabolically active cells after 72 h of culture were determined by the intensity of the purple color (formazan product) quantitatively measured by spectrophotometry at 590 nm wavelength (Fort et al., 2018). The absorbance measured directly correlates to percentage of viable cells.

The percentage of cell viability and inhibition were calculated through following formula:

\[
\text{% Cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of cells with vehicle solvent}} \times 100
\]

\[
\text{% Inhibition} = 100 - \text{% Cell viability}
\]

3. Results and discussion

The cytotoxicity of all extracts was dose-dependent, even though toxicity was dependent on the plant parts studied too (Manar et al., 2008). Medium lethality values of extracts ranged from 61.547 to 1277.543 µg/mL for P. harmala seeds (S) and P. harmala branches (B), respectively. In addition, P. harmala (S) caused 100% mortality of the brine shrimps within 24 h at 1000 µg/mL (Fig. 1). The outcomes of the brine shrimp lethality bioassay showed the P. harmala (S) extract to be extremely toxic to brine shrimp at 1000 µg/mL after 12 and 24 h exposure time. It was toxic at 500 µg/mL and 250 µg/mL after 12 and 24 h of exposure while slight toxicity was observed by the same extract at 62.5 µg/mL to 0.978 µg/mL as per Clarkson’s criteria. Slight toxicity was observed after 6 h of time exposure at the highest concentration tested, but no effect on lethality was recorded on lower concentrations after 6 h, and these outcomes are in accordance with earlier reports (Mackeen et al., 2000). The P. harmala (S) extract was highly toxic to shrimp larvae as per Clarkson’s toxicity criterion with median lethality value LC50 61.547 µg/mL obtained from logarithmic regression analysis (Fig. 2). Earlier reports showed the cytotoxic potential of plants as chief source of bioactives (Tawaha, 2006; Ali et al., 2011).

P. harmala roots (R) exhibited moderate toxicity to nauplii with 124 µg/mL LC50. This extract showed to be toxic to nauplii on 1000–250 µg/mL concentration after 12 and 24 h exposure time and slight toxicity was observed against brine shrimps on six different concentrations ranged from 125 µg/mL to 0.96 µg/mL and after 12–24 h (Fig. 1). Finally, 0.978 µg/mL concentration of extract was not much effective against brine shrimp larvae (Fig. 1). Results help to predict the presence of compounds with potential anti-cancer activity (Moshi et al., 2010).

P. harmala leaf (L) extract exhibited toxicity in concentrations higher than 500 µg/mL. Cytotoxicity was moderate at concentration range between 250 and 15.625 µg/mL. P. harmala (L) extract remained ineffective at concentrations lower than 7.81 µg/mL after 12 h. Slight toxicity was observed 24 h after exposure to concentration range between 7.81 and 1.96 µg/mL and 0.978 µg/mL concentration was nontoxic after 12–24 h (Fig. 1). The LC50 value of P. harmala (L) extract after 24 h of exposure was 567.082 µg/mL falling in low toxicity class according to Clarkson’s standard (Table 2). This suggests that the toxic properties of P. harmala are due to the seeds and roots and strengthen the speculation for capability to yield anticancer agents. (Tanko et al., 2007). The P. harmala branch (B) extract was slightly toxic for the brine shrimp after 12 h of exposure at seven different concentrations ranging between 15.625 and 1000 µg/mL and nontoxic at lower concentrations. After 24 h of exposure, the P. harmala (B) extract was moderately lethal on nine concentrations tested at 3.91–1000 µg/mL (Fig. 1). However, the extract showed a toxic effect at 1.96 µg/mL and 0.978 µg/mL after 24 h. P. harmala (B) extract was found to be

![Fig. 1. Brine Shrimp Lethality Test against logarithmic concentration of the plant extracts after 12 h and 24 h of exposure. Where MAz/F:12 = Lethality of Melia azedarach (fruit) after 12 h; MAz/L:12 = Lethality of Melia azedarach (Leaves) after 12 h; MAz/B:12 = Lethality of Melia azedarach (Branches) after 12 h; M.Al/L:12 = Lethality of Morus alba (Leaves) after 12 h; M.Al/B:12 = Lethality of Morus alba (Branches) after 12 h; P.Ha/L:12 = Lethality of Peganum harmala (Leaves) after 12 h; P.Ha/S:12 = Lethality of Peganum harmala (Seeds) after 12 h; P.Ha/R:12 = Lethality of Peganum harmala (Roots) after 12 h; Vin.C:12 = Lethality of Vincristine sulphate after 12 h; Etopside:12 = Lethality of Etopside after 12 h; K2Cr2O7:12 = Lethality of K2Cr2O7 (Potassium dichromate) after 12 h; M.Al/B:12 = Lethality of Melia azedarach (Leaves) after 12 h; P.Ha/L:12 = Lethality of Morus alba (Leaves) after 12 h; M.Al/L:12 = Lethality of Morus alba (Branches) after 12 h; P.Ha/S:12 = Lethality of Peganum harmala (Seeds) after 12 h; P.Ha/R:12 = Lethality of Peganum harmala (Roots) after 12 h; Vin.C:12 = Lethality of Vincristine sulphate after 12 h; Etopside:12 = Lethality of Etopside after 12 h; K2Cr2O7:12 = Lethality of K2Cr2O7 (Potassium dichromate) after 12 h.](image-url)
nontoxic with LC50 higher than 1000 μg/mL and was the least active with the highest LC50 1272.543 μg/mL among all assessed samples (Table 2). In few cases, thorough analysis of the cytotoxicity of plant parts allowed to highlight the cytotoxic compounds involved in the observed effects (Yvette et al., 2016).

*M. alba* (L) extract was toxic to brine shrimps at 500 μg/mL to 1000 μg/mL concentrations, but slight toxicity was observed at 250 μg/mL to 1.96 μg/mL, and 0.978 μg/mL concentration was not effective to show any significant lethality on brine shrimps after both 12 and 24 h exposure (Fig. 1). Overall, *M. alba* (L) extract was moderately lethal to shrimps with 310.327 μg/mL LC50 in the present study. 1000 μg/mL of *M. alba* (B) extract was toxic to brine shrimp nauplii after both 12 and 24 h exposure. Lethality values declined from 43 to 10 % with decreases in concentration from 500 μg/mL to 1.96 μg/mL. However, 0.978 μg/mL concentration of *M. alba* (B) extract revealed no significant lethality of nauplii after 12 and 24 h of exposure (Fig. 2). Based on calculated LC50 value of 1110.397 μg/mL after 24 h exposure, *M. alba* (B) extract was categorized as nontoxic to nauplii (Fig. 2). Other plant samples, however, showed no significant difference in percentage mortalities among different concentrations within the same species, signifying no brine shrimp lethality compared to that of control (Haque et al., 2009).

*M. azedarach* fruit (F) extract was toxic at concentrations of 250 μg/mL, 500 μg/mL and 1000 μg/mL. *M. azedarach* leaf (L) extract was toxic at 500 μg/mL and 1000 μg/mL. Slight toxicity against larvae was observed by *M. azedarach* (F) extract at concentration range 125–1.96 μg/mL after 12 h. The concentration 0.978 μg/mL was not lethal at that time. The 24 h exposure revealed slight toxicity on all tested concentrations in the range 0.978–125 μg/mL. A similar trend of lethality was observed for *M. azedarach* (L) extract after 12 and 24 h. The 0.978 μg/mL dose of *M. azedarach* (L) extract was not much lethal for brine shrimp nauplii after 12 h, but slight toxicity was reported after 24 h (Fig. 1). *M. azedarach* (F) and *M. azedarach* (L) extracts exhibited toxicity to shrimp larvae with 147.814 μg/mL and 310.327 μg/mL median lethality LC50 values estimated through logarithmic regression analysis (Table 2). These results extend the experimental findings on cytotoxicity of the fruit extract of *M. azedarach* (Khan et al., 2014). Potassium dichromate, Etoposide and Vincristine sulphate served as the positive control for BSLT and found to be extremely toxic as per Clarkson’s lethality criterion (Carballo et al., 2002). The LC50 values for the positive controls were 17.37, 3.598 and 2.13 μg/mL respectively, after 24 h of exposure (Fig. 2).

In the present study, all samples confirmed high to moderate cytotoxic activity on brine shrimps, ranging from 3.45 to 7.37 probit values on log 10 (conc) – 0.0097 to 3.0000 (Fig. 3). *P. harmala* (S) showed the highest activity on shrimps with 7.37 probit value, followed by *P. harmala* (R) with probit number 6.04 at log 10 (conc) 3.0, while the branches of same species had 5.1 probit (Fig. 3). However, the probit value of *P. harmala* (L) was about in the same range (5.39). *P. harmala* (S) was found to be significant with probit value range 3.72–7.37 on log10 (c) – 0.0097 to 3.0, while *P. harmala* (R) had less variations with 3.59–6.04 probit values (Fig. 3). Conversely, *P. harmala* (L) and *P. harmala* (B) were not much active with probit value ranges 3.45–5.39 and 3.52–5.1, respectively. *M. azedarach* (F) also revealed significant results with 5.92 probit value of mortality percentage, different from that of *M. azedarach* (L) with 5.52 at log 10 (conc) 3.0. No significant difference was found between *M. alba* (L) and *M. alba* (B) at log10 (c) – 0.0097, but both samples showed a clear difference in cytotoxic activity on the shrimp assay at log10 (c) 3.0 with probit values from 5.39 to 5.15 (Fig. 3).

Maximum mortality for general toxicity via BSLT was observed with 1000 μg/mL concentration whereas 0.978 and 1.96 μg/mL were the least active concentrations. This explains that degree of toxicity was proportional to concentrations of samples (Tanko et al., 2007). Furthermore, the brine shrimp lethality of plant bioactive correlates reasonably fine with their cytotoxic potential, thus providing novel therapeutic candidates for numerous maladies. Logarto et al. (2001) verified that there was a strong correlation (P < 0.05) in LC50 of BSLT and LD50 of acute oral toxicity in mice. This substantiates that brine shrimp lethality is valuable for the examination of plant extracts, to estimate their toxicity level. From a pharmacological perspective, a good association has been found with the BSLT used to discover antitumoral compounds from plant extracts (Billah et al., 2013). In our experimental conditions, trend of median lethal concentration, LC50 after 24 h, of the methanolic extracts was:

**Table 2**

| Sample               | Part used       | Cytotoxicity Regression Equation | Antitumor Activity Regression Equation | Toxicity class  |
|----------------------|-----------------|---------------------------------|---------------------------------------|-----------------|
| Melia azedarach      | Fruits          | y = 9.131In(x) + 4.382          | y = 15.63In(x) - 27.17                  | Moderately toxic|
| Melia azedarach      | Leaves          | y = 8.042In(x) + 3.858          | y = 10.25In(x) - 9.303                  | Moderately toxic|
| Morus alba           | Leaves          | y = 7.675In(x) + 2.850          | y = 13.62In(x) - 22.99                  | Moderately toxic|
| Morus alba           | Branches        | y = 6.494In(x) + 4.461          | y = 7.112In(x) - 7.583                  | Non toxic       |
| Peganum harmala      | Leaves          | y = 7.530In(x) + 2.256          | y = 12.05In(x) - 22.85                  | Low toxic       |
| Peganum harmala      | Seeds           | y = 11.82In(x) + 1.304          | y = 15.89In(x) - 12.87                  | Highly toxic    |
| Peganum harmala      | Branches        | y = 6.231In(x) + 5.456          | y = 8.385In(x) - 20.75                  | Non toxic       |
| Peganum harmala      | Roots           | y = 10.35In(x) + 0.091          | y = 14.24In(x) - 21.37                  | Moderately toxic|
| Vincristine C         | -               | y = 9.406In(x) + 42.89          | y = 3.130In(x) + 78.89                  | Highly toxic    |
| Etoposide            | -               | y = 9.669In(x) + 37.62          | y = 3.087In(x) + 78.77                  | Highly toxic    |
| K2Cr2O7              | -               | y = 10.81In(x) + 19.14          | -                                      | Highly toxic    |
P. harmala (B) > M. alba (B) > P. harmala (L) > M. alba (L) > M. azedarach (F) > P. harmala (R) > P. harmala (S).

Although some species were more active after 24 h than after 12 h, in few cases there was no remarkable variation with time in the potential of the extracts (Ullah et al., 2013). After 24 h of exposure, bioactivity of the extracts amplified significantly because sensitivity of brine shrimps increased. At this phase, nauplii possess II instar and show their high sensitivity to the treatment. The demise of the nauplii may be due to the influence of starvation or plant extract (Ali et al., 2013). To confirm the lethality of the plant extracts, control having only nauplii was also tested (Nondo et al., 2011). In any situation, hatched nauplii can stay alive up to 2 days without nutrition as yolk sac is sufficient to nourish. Normal yeast is supplemented as food of nauplii on the second day (Mbawmbo et al., 2007). The effects of the plant species are consistent with the phytochemical data of these plants as a source of active cytotoxic and antitumor compounds (Bastos et al., 2009).

So, our findings indicated BSLT as convenient bioassay to screen natural products in drug discovery process (Cyrus et al., 2008). The LC50 values of plant extracts (at 24 h) were achieved through a plot of proportion of the shrimps killed alongside the concentrations of extracts and best-fit line was acquired from the statistics by means of regression analysis to calculate LC50 value (Roy et al., 2011).

In the present study, the potato disc bioassay was used as anti-tumor prescreen against plant crude extracts. Crown gall is a plant disease characterized by cell hyperplasia and hypertrophy caused by the gram negative bacterium Agrobacterium tumefaciens. It was shown that A. tumefaciens At 10 strain was very active in tumor production with 37.33 ± 1.05 tumor count per disc (Table 3). The bacterium has a large tumor-inducing plasmid that possesses a T-DNA that alters normal cells into self-directed tumor cells (Ahmad et al., 2016). Significant activity in the PDA was recorded in six plant extracts while two extracts were not effective (Fig. 4). The use of an antitumor prescreen assay, by means of plant systems, possesses several benefits as an option to animal systems for exploration of novel anticancer leads (Ayaz et al., 2016).

M. azedarach (F) extract exhibited 87.60 and 73.87% inhibition of At 10 induced development of crown gall tumors on Solanum at concentrations of 1000 and 500 μg/mL, respectively. Activity decreased with decrease in concentration and the lowest activity (22.98%) was observed with M. azedarach (F) at 15.625 μg/mL. M. azedarach (F) extract caused 50% inhibition (IC50) of crown-gall tumors at concentration of 138.517 μg/mL (Fig. 5). M. azedarach (L) inhibited the formation of crown-gall tumors by 54.35, 45.14, 36.75, 29.36, 27.91 and 22.18% at concentrations of 1000–15.625 μg/mL (Fig. 4). The LC50 of crown-gall formation observed with M. azedarach (L) was found to be 325.596 μg/mL (Table 3). A relationship exists between concentration of samples and ability to restrain crown gall tumor development on potato discs (Khan et al., 2016).

M. alba (L) extract showed 70.96% inhibition at a dose of 1000 μg/mL. The inhibition potential declined from 65.15% to 46.26% with decrease in concentration from 500 to 125 μg/mL (Fig. 4). Tumor inhibition percentage was found to be<30% at concentration lower than 62.5 μg/mL and the lowest inhibition percentage was found to be 19.50% at 15.625 μg/mL of M. alba (L) extract. M. alba (B) was found to be a poor inhibitor of At induced crown gall tumors and exhibited 43.45% inhibition at 1000 μg/mL of extract concentration. The inhibition percentage extensively decreased with a reduction of 30% at concentrations < 250 μg/mL and 14.25% was the lowest activity obtained. IC50 values determined through logarithmic regression analysis of M. alba (L) and M. alba (B) extracts were 198.93 μg/mL and 3283.28 μg/mL, respectively, which suggested that different parts of the same plant showed huge difference in the inhibition potential of tumors on potato discs (Table 2).

Results showed that the extract of P. harmala (S) inhibited tumor growth in a concentration dependent trend (Table 3). Significant tumor inhibition was observed at five different concentrations from 1000 to 62.5 μg/mL but not at 31.25 and 15.625 μg/mL. P. harmala (S) showed very promising results with 94.98% inhibition of crown gall tumors at 1000 μg/mL extract (Fig. 4). Minimum (27.56%) tumor inhibition was recorded by 15.625 μg/mL of the same extract (Table 3). The extract of P. harmala (L) demonstrated 62.55% inhibition of the tumors at 1000 μg/mL whereas 500 μg/mL extract was able to cause 52.34% inhibition of crown gall tumors. Inhibitory effects of 250 μg/mL P. harmala (L) were 46.53% and the lowest activity (14.68%) was shown by the lowest concentration (15.625 μg/mL) tested among all seven concentrations tested (Fig. 4). P. harmala (R) showed significant inhibitory effect on the crown-gall tumor development as evident by the maximum inhibition of 78.22% at 1000 μg/mL. On the other hand, P. harmala (B) was found to be poorly active because of the maximum inhibition of 34.93% at the same concentration. The inhibitory activity of 64% and 32.41% at 500 μg/mL was observed with the crude extracts of P. harmala (R) and P. harmala (B), respectively. Percentage inhibition > 50% was exhibited by P. harmala (R) on concentrations > 125 μg/mL, but lower concentrations of the extract were not much effective against tumors. Nevertheless, 125 μg/mL of the P. harmala (B) extract was not sufficient to cause 20% inhibition of crown gall tumors. P. harmala (B) was considered almost inactive at 15.625 μg/mL concentration with the least value of percentage inhibition (2.17%) achieved in current study.

The differences in the IC50 values of four different parts of the P. harmala indicated the evident difference in potential of inhibition on crown gall tumor. The maximum LC50 value (4617.86 μg/mL) was shown by P. harmala (B) while the lowest LC50 (52.278 μg/mL) was reported by the P. harmala (S) (Fig. 5). The LC50 of P. harmala (R) (150.196 μg/mL) was lower than the P. harmala (S) one, but higher than that of P. harmala (L) (IC50 = 422.269 μg/mL). Vincristine and Etoposide (positive controls) caused 100% cancer inhibition at 1000–500 μg/mL concentrations. No significant difference in bioactivity between Etoposide and Vincristine was observed in current study, though a slight variation in inhibition along different concentrations was observed. The minimum activity of Vincristine was registered as 88.21% inhibition at 15.625 μg/mL concentration. IC50 values determined through logarithmic regression for Etoposide and Vincristine were 9 and 9.8 μg/mL, respectively (Fig. 5).
Table 3
Inhibition of crown gall tumors by the plant extracts.

| Test material | Dose (µg/mL) | Tumor count per disc* | Inhibition of tumor [%] | IC50 (µg/mL) |
|---------------|--------------|------------------------|-------------------------|-------------|
| Negative control | –            | 37.33 ± 1.05           | 100.00                  |             |
| Vincristine C | 1000         | 0.00 ± 0.00            | 100.00                  | 9.8         |
| 500           | 0.00 ± 0.00  | 100.00                  |                         |             |
| 250           | 1.79 ± 0.59  | 95.20                  |                         |             |
| 125           | 2.13 ± 0.93  | 94.29                  |                         |             |
| 62.5          | 3.41 ± 0.72  | 90.87                  |                         |             |
| 31.25         | 3.93 ± 0.49  | 89.47                  |                         |             |
| 15.625        | 4.40 ± 1.21  | 88.21                  |                         |             |
| Etoposide     | 1000         | 0.00 ± 0.00            | 100.00                  | 9.00        |
| 500           | 0.00 ± 0.00  | 100.00                  |                         |             |
| 250           | 1.93 ± 0.21  | 95.20                  |                         |             |
| 125           | 2.85 ± 0.84  | 92.37                  |                         |             |
| 62.5          | 3.39 ± 0.30  | 90.92                  |                         |             |
| 31.25         | 4.08 ± 0.30  | 89.07                  |                         |             |
| 15.625        | 4.25 ± 1.17  | 88.62                  |                         |             |
| M.Az/F        | 1000         | 4.63 ± 1.15            | 87.50                   | 138.517     |
| 500           | 9.83 ± 9.2   | 73.67                  |                         |             |
| 250           | 17.89 ± 2.06 | 52.08                  |                         |             |
| 125           | 21.95 ± 2.71 | 41.20                  |                         |             |
| 62.5          | 24.78 ± 3.61 | 33.62                  |                         |             |
| 31.25         | 28.93 ± 0.94 | 27.86                  |                         |             |
| 15.625        | 28.75 ± 1.79 | 22.98                  |                         |             |
| M.Az/L        | 1000         | 12.84 ± 1.40           | 65.60                   | 325.596     |
| 500           | 17.04 ± 1.39 | 54.35                  |                         |             |
| 250           | 20.48 ± 2.05 | 45.14                  |                         |             |
| 125           | 23.61 ± 2.78 | 36.75                  |                         |             |
| 62.5          | 26.37 ± 3.31 | 29.36                  |                         |             |
| 31.25         | 26.91 ± 2.28 | 27.91                  |                         |             |
| 15.625        | 29.05 ± 4.01 | 22.18                  |                         |             |
| M.Al/L        | 1000         | 10.84 ± 0.63           | 70.96                   | 198.93      |
| 500           | 13.01 ± 1.83 | 65.15                  |                         |             |
| 250           | 17.21 ± 1.50 | 53.90                  |                         |             |
| 125           | 20.06 ± 0.27 | 46.26                  |                         |             |
| 62.5          | 27.67 ± 0.75 | 25.88                  |                         |             |
| 31.25         | 28.32 ± 1.05 | 24.14                  |                         |             |
| 15.625        | 30.05 ± 1.61 | 19.50                  |                         |             |
| M.Al/B        | 1000         | 21.11 ± 2.10           | 43.45                   | 3283.28     |
| 500           | 23.65 ± 2.91 | 36.65                  |                         |             |
| 250           | 25.98 ± 1.54 | 30.40                  |                         |             |
| 125           | 27.76 ± 2.27 | 25.64                  |                         |             |
| 62.5          | 29.65 ± 1.79 | 20.57                  |                         |             |
| 31.25         | 31.23 ± 2.26 | 16.34                  |                         |             |
| 15.625        | 32.01 ± 1.92 | 14.25                  |                         |             |
| P.Ha/L        | 1000         | 13.98 ± 0.98           | 62.55                   | 422.269     |
| 500           | 17.79 ± 1.57 | 52.34                  |                         |             |
| 250           | 19.96 ± 1.43 | 46.53                  |                         |             |
| 125           | 26.21 ± 1.49 | 29.79                  |                         |             |
| 62.5          | 29.14 ± 2.97 | 21.94                  |                         |             |
| 31.25         | 30.05 ± 1.77 | 19.50                  |                         |             |
| 15.625        | 31.85 ± 1.03 | 14.68                  |                         |             |
| P.Ha/S        | 1000         | 1.87 ± 0.57            | 94.99                   | 52.278      |
| 500           | 4.72 ± 0.86  | 87.36                  |                         |             |
| 250           | 10.04 ± 0.45 | 73.10                  |                         |             |
| 125           | 13.45 ± 1.60 | 63.97                  |                         |             |
| 62.5          | 15.3 ± 1.22  | 59.01                  |                         |             |
| 31.25         | 21.93 ± 1.81 | 41.25                  |                         |             |
| 15.625        | 27.04 ± 0.30 | 27.56                  |                         |             |
| P.Ha/B        | 1000         | 24.29 ± 1.25           | 34.93                   | 4617.86     |
| 500           | 25.23 ± 2.06 | 32.41                  |                         |             |
| 250           | 26.72 ± 1.73 | 28.42                  |                         |             |
| 125           | 30.06 ± 3.06 | 19.47                  |                         |             |
| 62.5          | 32.62 ± 4.29 | 12.62                  |                         |             |
| 31.25         | 34.31 ± 4.20 | 8.09                   |                         |             |
| 15.625        | 36.52 ± 2.84 | 2.17                   |                         |             |
| P.Ha/R        | 1000         | 8.13 ± 0.58            | 78.22                   | 150.196     |
| 500           | 13.44 ± 1.59 | 64.00                  |                         |             |
| 250           | 15.39 ± 0.14 | 58.77                  |                         |             |
| 125           | 17.66 ± 1.39 | 52.69                  |                         |             |
| 62.5          | 25.83 ± 0.32 | 30.81                  |                         |             |
| 31.25         | 26.97 ± 1.16 | 27.75                  |                         |             |
| 15.625        | 30.03 ± 0.90 | 19.56                  |                         |             |

*mean of twelve replicates.

Where M.Az/F = Melia azedarach (fruit); M.Az/L = Melia azedarach (Leaves); M.Al/L = Morus alba (Leaves); M.Al/B = Morus alba (Branches); P.Ha/L = Peganum harmala (Leaves); P.Ha/S = Peganum harmala (Seeds); P.Ha/B = Peganum harmala (Branches); P.Ha/R = Peganum harmala (Roots).
Animals and plants share few common mechanisms of tumorigenesis including the intracellular integration of exogenous genetic material; therefore, it might be predicted that some anticancer compounds prevent tumor formation and development in different organisms (Sahreen et al., 2015).

This work has emphasized the significance of brine shrimp bioassay to assess the biological activity of plants frequently used by several societies and ethnic groups for their therapeutic indications. Several reports have tried to associate the toxicity of phytochemicals from medicinal plants to *Artemia salina* with bioactivities like antiproliferative (Salawu et al., 2017), antiangiogenic (Ayaz et al., 2016), anticarcinogenic (Kumar et al., 2014) and antitumor (Wang et al., 2017) potentials, among others.

Researchers have systematically performed this bioassay in initial evaluation of extracts recognized as antitumor agents (Arcanjo et al., 2012). This implies a positive correlation of brine shrimp lethality with antitumor activity in the development of new anticancer lead drugs from plants.

Flavonoid and phenolic contents of natural products are well known for many biological properties including antioxidant, anticarcinogenic, antineovascularization and antiproliferative activities and, consequently, they are considered as chemopreventive agents (Baba and Malik, 2015). Flavonoids can scavenge reactive oxygen species (ROS) in oxidative stress, condition that might promote carcinogenesis and/or angiogenesis. Likewise, phenolics are well known for their multitarget biological activity (Balasundram et al., 2006). Numerous preclinical studies suggested a protective role of phenolics in the prevention of colon, breast, ovary, prostate, lung and endometrium cancers (Galati and Brien, 2004; Baghel et al., 2012). Total phenolic content of samples was estimated through linear regression equation of calibration curve and expressed as gallic acid equivalent (mg GAE/g dry mass) (Fig. 8: Supplementary data). Results showed that the extracts of *M. azedarach* (L) and (B) were rich in phenolics with 543 ± 0.04 and 287 ± 0.01 mg GAE/g, respectively. P. harmala (S) extract also contained high levels of phenolics (271 ± 0.02 mg GAE/g). Results were in agreement with the previous study by Hassan et al. (2014) on numerous plant extracts screened for antiangiogenic potential and phenolic content. It was found that extracts of *M. azedarach* (L) and (B) were rich in phenolics with 543 ± 0.04 and 287 ± 0.01 mg GAE/g, respectively. *P. harmala* (S) extract also contained high levels of phenolics (271 ± 0.02 mg GAE/g). Results were in agreement with the previous study by Hassan et al. (2014) on numerous plant extracts screened for antiangiogenic potential and phenolic content. It was found that extracts of *M. azedarach* (L), *P. harmala* (B) and *P. harmala* (R) were poor of phenolics. On the other hand, *P. harmala* (L) and (F) extracts of *M. azedarach* exhibited moderate phenolic levels (113 ± 0.06 mg GAE/g and 107 ± 0.03 mg GAE/g, respectively). Phenolic compounds behave as metal chelating agents in the oxidative systems, thereby neutralizing free radical species. A number of evidence (Yeldandi et al., 2000; Galati and Brien, 2004) have proposed that reactive oxygen species (ROS) are crucial signaling molecules that activate cell growth and proliferation, and ultimately promote tumorigenesis and angiogenesis (Wang et al., 2017).

![Fig. 4. Percentage Inhibition of Tumor at seven different concentrations.](image)

![Fig. 5. Antitumor activity LC50 and R² value determined through logarithmic regression analysis. Where M.Az/F = Melia azedarach (fruit); M.Az/L = Melia azedarach (Leaves); M.Al/L = Morus alba (Leaves); M.Al/B = Morus alba (Branches); P.Ha/L = Peganum harmala (Leaves); P.Ha/S = Peganum harmala (Seeds); P.Ha/B = Peganum harmala (Branches); P.Ha/R = Peganum harmala (Roots); Vin.C = Vin-cristine sulphate; Etopside = Etopside; K2Cr2O7 = K2Cr2O7 (Potassium dichromate).](image)
Total flavonoid content of samples was expressed in quercetin equivalent (QE) and calculated through regression analysis (Fig. 8: Supplementary data). Numerous studies reported the capability of flavonoids to inhibit cancer cell growth in vitro (Moghaddam et al., 2012; Delmalle et al., 2006; Chidambaram et al., 2012). Flavonoids possess valuable antioxidant and antitu-
cancer potentials, and act as antiangiogenic agents through key mechanisms of carcinogenesis. Metabolic activation of (pro)car-
cinogens is hindered by flavonoids in early phases of carcino-
genesis, while in progression stages, angiogenesis and metastases are inhibited due to induction of apoptosis (Clere et al., 2011). P. harm-
ala (S) extract exhibited the highest levels of flavonoids, i.e., 449 ± 0.01 mg QE/g. Extracts of M. alba (L) and (B) showed high levels of flavonoids too, 291 ± 0.00 mg QE/g and 164 ± 0.03 mg QE/g, respectively. P. harmala (R) and (B) extracts exhibited low total flavonoid contents (28 ± 0.01 mg QE/g and 32 ± 0.06 mg QE/g, respectively) (Fig. 8: Supplementary data). P. harmala (L) con-
tained moderate levels of total flavonoids with 73 ± 0.02 mg QE/g. Finally, M. azedarach (F) and (L) exhibited very low contents of total flavonoids, 12 ± 0.02 mg QE/g and 23 ± 0.00 mg QE/g, respectively (Fig. 8: Supplementary data). The cytotoxic and antitumor activi-
ties of plant extracts could be, at least in part, attributed to their high flavonoid content (Dai et al., 2013). Many studies have reported the occurrence of phenolic compounds and complex prenylated flavones in plant extracts, and thus their cytotoxic and antitumor activities could be ascribed to additive and/syner-
gistic effects of bioactive phytochemicals (Elkashak et al., 2017). In recent addition to their capability to inhibit oxidation of free radicals as well as antioxidant activity, polyphenols are also capable of inhibiting ROS generation in biological systems (Elkashak et al., 2017). Consequently, polyphenols exert a pivotal role in counteracting angiogenesis, in addition to their ability to inhibit oxidation of free radicals as natural cytoprotective agents (Elkashak et al., 2017). In recent times, a huge number of studies have been focused on phenolics and flavonoids for the reduction of ROS generation in biological systems (Do et al., 2014). Indeed, oxidative stress generated by ROS plays a very critical role in the pathogenesis of chronic, degener-
ative diseases including cancer (Abozed et al., 2014).

3.1. Isolation of PH-HM-16

The five groups of elution were tested for their bioactivities after elutions and Group 2 was found to have significant cytotoxic potential with LC50 value of 25.84 µg/mL and was considerably effective to inhibit crown gall tumorgenesis with IC50 value of 28.36 ± 0.03 µg/mL. All the three subgroups of group 2 were again tested for their bioactivities and Group 2b was found to be the most potent group with respect to its cytotoxic potential (IC50 value = 16.52 µg/mL) and antitumor activity (IC50 = 20.67 µg/mL) compared with Group 2a and Group 2c (Fig. 9: Supplementary data). The LC50 value for cytotoxic potential of Group 2a was 30.46 µg/mL and for Group 2c it was 28.14 µg/mL. While antitumor potential of Group 2a (IC50 = 38.10 µg/mL) and Group 2c (IC50 = 33.34 µg/mL) was also less than Group 2b, the latter was selected on the basis of high bioactivity to further check for the type of chemical constituents present therein (Fig. 9: Supplementary data). HPLC anal-
ysis leads to total of 63 elutions were collected and acquired Group 2b.A, Group 2b.B, Group 2b.C and Group 2b.D (Fig. 9: Supplementary data). All the groups were again checked for their bioactivities and Group 2b.A (elutions 4–13) was found to have significant cyto-
toxic (LC50 = 12.80 µg/mL) and antitumor (IC50 = 14.16) potentials. Finally, from Group 2b.A, pure colorless solid needles of the compound PH-HM-16 were isolated by recrystallization by using methanol:acetone mixture from the eluates obtained. Summary of the PH-HM-16 isolated is given in the Fig. 9: Supplementary data.

3.2. Identification and structure elucidation of isolated compound PH-
HM-16

3.2.1. Physical characteristics of PH-HM-16.

PH-HM-16 was colorless, odorless, in form of needles with melting point range of 210 °C-214 °C. PH-HM-16 compound was soluble in acetone.

3.2.2. Spectroscopic analysis

Mass spectra of PH-HM-16 was recorded in the positive ioniza-
tion mode on mass spectrometer. The molecular formula was assigned as C11H12N2O ([M + H]+, m/z 189.07683; calculated 189.23) by HRESIMS (Fig. 6a). ESI-MS spectral analysis of PH-HM-16 revealed dynamic energy of MS2 from protonated PH-HM-16 [M + H]+ + at 189.07683 m/z afforded the fragment ion at m/z 171.12705 by the loss of one water molecules (18 Da) (Fig. 6f). Consequently, a carbon–carbon double bond among C-1 and C-2 was formed through elimination reaction (Fig. 6f). The product ion at 154.67308 m/z was formed by elimination of ammonia (17 Da, NH3) (Fig. 6a).

FTIR (Fourier Transform Infrared spectroscopy) spectrum of the isolated PH-HM-16 was determined by using KBr disk method on the IR spectrophotometer (BUCK SCIENTIFIC). The stretching vibra-
tion of hydroxyl group (3421 cm⁻¹) and olefinic groups (1508 cm⁻¹) was identified from the IR spectrum (Fig. 6b). The stretching for the hydroxyl group in the compounds appears in the range of 3287 cm⁻¹ to 3487 cm⁻¹ (Barud et al., 2013). The C- N in the structure of active principle was confirmed by FTIR which showed the characteristic absorbance peak spectrum at 1085 cm⁻¹ (Fig. 6b) as this is the characteristic band range for the C-N group in the organic compounds (Oliveira et al., 2016).

1H NMR. Proton nuclear magnetic resonance spectra of PH-HM-16 in DMSO confirmed aromatic hydrogens in the structure by res-
onances at multiplet peaks δH 7.399–7.314 (m, H-7, H-8, H-9, H-10) indicated the presence of olefinic bond (Fig. 6c). Chemical shift for the olefinic bond was also in this range in the previous reports which validate the current results (Timmers et al., 2012). One tri-
plet proton signal appeared at δH 5.1 (t, H-1) was attributed to the hydroxymethylene proton (Table 4). A range of isolated com-
ponds clearly established that δH of the hydroxymethine proton was appeared in this range of chemical shift so this strengthen the interpretation of current spectrum (Montenegro et al., 2019). Proton NMR showed one proton signal at δH 4.78 (s, br, H5a, H5b) and three multiplet peaks centered at 2.15 (m, H-2a), 2.56 (m, H-2b) due to methylene and 3.50 (m) is assigned to the H-3a and H-3b protons (Fig. 6c).

13C NMR spectroscopic experiment (DMSO) showed eleven carbon atoms with eight methylene and three methine resonances (Fig. 6f). It also showed the presence of a carbon (C-1) bearing a hydroxyl group at δC 72.947 which was attributed to confirmation of hydroxyl group in the isolated compound (Fig. 6d). 13C NMR peaks at the range of 70–79 could fairly be due to the carbon atom bearing hydroxyl group in the compound so this coincides with current spectrum (Sanches, 2005). The peaks observed in the downfield of spectra in the range δC 120–140 ppm can be assigned to greatly deshielded carbon atoms of the compound with C = C double bond (Edilu et al., 2015). Therefore, the presence of double bonds was supported by the appearance of three methane carbon signal at δC 120.896 (C-6), 143.715 (C-11) and resonance at 164.524 due to C-13 in the structure (Table 4). The position of ben-
zeine ring is confirmed by the appearance of 13C NMR spectrum at δC 124.313 ppm, C-8 (δC 126.799), C-9 (127.766 ppm) and C-10 (δC 129.423) indicating sp² hybridized aromatic carbon in the compo-
und (Fig. 6d). The rests of the carbons were appeared in aliphatic region at δC 30.508 (C-2), 48.263 because of C-3 in compound and δC at 49.952 ppm (C-5) chemical shifts with distinguishable
peaks coincide with HSQC correlation thus identified as PH-HM-16 (Fig.0.11).

HSQC. The heteronuclear single quantum correlation or heteronuclear single quantum coherence experiment revealed the number of protons in the compound attached to specific carbon atoms (Fig. 6f). HSQC of the isolated compound PH-HM-16 is given in the Fig. 6e. It revealed that C-13 with resonance at $\delta C = 164.524$ ppm and C-6 with chemical shift $\delta C = 120.896$ ppm are not attached to any of the proton (Fig. 6f). Similarly, resonance at $\delta C = 143.715$ ppm in the spectrum due to C-11 was not linked with any of hydrogen in the HSQC spectra (Fig. 6e).

Hence this confirms that C-6, C-11 and C-13 might be the quaternary carbon in the structure of PH-HM-16 (Fig. 6f). Carbon-2 ($\delta C = 30.508$ ppm) is methylene carbon bonded to H-2a (m, $\delta H = 2.15$) and H-2b (m, $\delta H = 2.56$). Likewise, C-3 ($\delta C = 48.263$ ppm) is also attached with H-3a and H-3b protons (m, $\delta H = 3.50$ ppm) while the rest of carbon atoms in the molecule of PH-HM-16 are CH revealed in HSQC correlation (Fig. 6e).

4. Anticancer potential of PH-HM-16

Isolated compound PH-HM-16 was evaluated for its cytotoxic potential against six human cancer cell lines namely breast cancer
human myeloid leukemia (HL-60), prostate cancer (PC-3), human gastric cancer (SGC-7901), human colorectal cancer (HCT-116) and human lung adenocarcinoma (A549) cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. The cytotoxicity of isolated compound PH-HM-16 was examined at concentration ranging from 31.25 μg/mL to 500 μg/mL. The absorbance of all the samples was highly dependent on the concentration of compound used (Fridlender et al., 2015). Dose-related curves of percentage of cell viability (%) at each concentration was calculated by absorbance of each sample to negative control (Mathi et al., 2015). Results revealed that compound PH-HM-16 was significantly active against prostate cancer (PC-3) and breast cancer (MCF-7) cell lines. The minimum absorbance of 0.116 nm and 0.123 nm was measured by the MCF7 and PC-3 cells, respectively, at 500 μg/mL of compound PH-HM-16, i.e., 14.181% and 15.037% cell viability, respectively (Fig. 10: Supplementary data). The value of absorbance increased from 0.250 to 0.371 nm in prostate cancer cell line and 0.247 to 0.390 nm in breast cancer cell line (Mathi et al., 2015). Dose-related curves of percentage of cell viability (%) at each concentration was calculated by absorbance of each sample to negative control (Mathi et al., 2015). Results revealed that compound PH-HM-16 was significantly active against prostate cancer (PC-3) and breast cancer (MCF-7) cell lines. The minimum absorbance of 0.116 nm and 0.123 nm was measured by the MCF7 and PC-3 cells, respectively, at 500 μg/mL of compound PH-HM-16, i.e., 14.181% and 15.037% cell viability, respectively (Fig. 10: Supplementary data). The value of absorbance increased from 0.250 to 0.371 nm in prostate cancer cell line and 0.247 to 0.390 nm in breast cancer cell line (Mathi et al., 2015). Dose-related curves of percentage of cell viability (%) at each concentration was calculated by absorbance of each sample to negative control (Mathi et al., 2015). Results revealed that compound PH-HM-16 was significantly active against prostate cancer (PC-3) and breast cancer (MCF-7) cell lines. The minimum absorbance of 0.116 nm and 0.123 nm was measured by the MCF7 and PC-3 cells, respectively, at 500 μg/mL of compound PH-HM-16, i.e., 14.181% and 15.037% cell viability, respectively (Fig. 10: Supplementary data).

Similar trend in percentage of cell viability was observed in other cell lines (Fig. 7A). This is in strong correlation with toxicological profiles for such compounds and also with their in vivo toxicity (Fort et al., 2018). Significant cell growth inhibition was observed against prostate (PC-3) and breast (MCF7) cancer cell lines after treatment with PH-HM-16, with maximum inhibition of 84.96% and 85.81% at the dose of 500 μg/mL, and lowest inhibition of 54.65% and 48.29% at 31.25 μg/mL concentration, respectively (Fig. 7B). PH-HM-16 was most effective against PC3 cell line with IC50 value of 17.63 μg/mL followed by MCF7 with IC50 value of 41.81 μg/mL. This strongly agrees with a very recent report which clearly indicated significant in vitro anticancer activity by increasing concentration (Nguyen et al., 2020). Isolated compound PH-HM-16 showed moderate activity on human myeloid leukemia (HL-60) cell line. The minimum cell viability observed in this cell line was 23.59% with 0.193 nm absorbance at the dose of 500 μg/mL of PH-HM-16 (Fig. 10: Supplementary data). Gradual decrease in the dose level of the compound to 31.25 μg/mL increase the value of cell viability to 71.39% with absorbance of 0.584 nm (Fig. 7A). Percentage inhibition of human myeloid leukemia cells observed was in the range 28.61% to 76.40% at the concentration of 31.25 μg/mL to 500 μg/mL, with IC50 value of 68.77 μg/mL indicating a moderate efficacy of the compound (Table 5). Hence, anticancer activity of compound was highly concentration dependent (Solowey et al., 2014). Compound PH-HM-16 was weakly effective on human colorectal tumor cells (HCT-116), with maximum percentage inhibition 75.42% and absorbance value of 0.201 nm at the maximum dose of compound used (500 μg/mL), and the lowest inhibition observed was 37.41% with absorbance value of 0.512 nm at the lowest dose of the compound (31.25 μg/mL) used in the experiment (Fig. 7A). IC50 value against HCT-116 cells was observed to be 71.54 μg/mL (Table 5). HCT-116 cell line was also considered as sensitive in previous studies reported for anticancer activity of compounds isolated from Drechslera rostrata and Eurotium tonophilum (Alasmary et al., 2018). PH-HM-16 did not show significant inhibitory activity against human gastric cancer (SGC-7901) and human lung adenocarcinoma epithelial (A549) cell lines. Compound PH-HM-16 exhibited the lowest cell growth inhibition (15.16%) at the absorbance value of 0.694 nm at 31.25 μg/mL concentration of the compound (Fig. 10: Supplementary data). On the other hand, compound PH-HM-16 at dose of 31.25 μg/mL inhibited the SGC-7901 tumor cell growth by 36.92% (Fig. 7B). These results

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### Table 4

| Position | 13C δC (ppm) | 1H δH (ppm) | HSQC |
|----------|--------------|-------------|------|
| 1        | 72.947       | 5.10 (t)    | CH   |
| 2        | 30.508       | 2.15 (m)    | CH₂  |
| 3        | 48.263       | 3.50 (m)    | CH₃  |
| 4        | 49.952       | 4.78 brs    | CH₃  |
| 5        | 120.896      | –           | C    |
| 6        | 124.313      | 7.39 (m)    | CH   |
| 7        | 126.799      | 7.33 (m)    | CH   |
| 8        | 127.766      | 7.31 (d)    | CH   |
| 9        | 129.423      | 7.37 (m)    | CH   |
| 10       | 143.715      | –           | C    |
| 11       | 164.524      | –           | C    |

### Table 5

| Cell lines | IC50 | Regression Equation | R² |
|------------|------|---------------------|----|
| HL-60      | 68.77| y = 15.838ln(x) - 17.008 | 0.7548 |
| PC-3       | 17.63| y = 10.512ln(x) + 19.834 | 0.9898 |
| SGC-7901   | 111.89| y = 12.593ln(x) - 9.4079 | 0.9698 |
| MCF-7      | 41.81| y = 13.104ln(x) + 0.9817 | 0.9646 |
| HCT116     | 71.54| y = 13.845ln(x) - 9.1215 | 0.97 |
| Lung A549  | 176.04| y = 18.783ln(x) - 47.122 | 0.9734 |

Where HL-60 = Human myeloid leukemia cell line; PC-3 = Prostate cancer cell line; SGC-7901 = Human gastric cancer cell line; MCF-7 = Breast cancer cell line; HCT-116 = Human colorectal cancer cell line; Lung A549 = Human lung adenocarcinoma cell line.
coincide with previous report about the anticancer potential of sesquiterpenoids isolated from the Solanum lyratum and also tested against stomach gastric cancer cell lines (Chen et al., 2017). The IC50 value of PH-HM-16 compound was not significant against SGC-7901 (111.89 μg/mL) and A549 (176.04 μg/mL) cancer cell lines (Table 5). These result implies that compound PH-HM-16 has significant cytotoxic activity against some cancer cell lines. The bioassays for the bioactive compound isolated clearly indicates that it might hold a promising perspective in the future for the development of anticancer agents.

5. Conclusions

In conclusion, among the eight plant extracts tested, P. harmala (seeds + roots), M. azedarach (fruits) and M. alba (leaves) were the most biologically active extracts with significant antitumor and cytotoxic effects which might be due to presence of phytochemicals, including flavonoids and phenolics. Bioactivity guided isolation of extracts led us to the isolation of PH-HM-16 from fraction P. harmala seeds. The results demonstrated significant anticancer activity against six human cancer cell lines assessed through MTT cell growth inhibition assay. PH-HM-16 was most effective against human breast cancer (MCF7) cell line with IC50 value of 17.63 μg/mL followed by breast cancer (MCF7) cell line with IC50 value of 41.81 μg/mL. Hence, this compound shows a promising potential to be chemically standardized or developed into the food supplements or pharmaceuticals or as adjuvant agents for the chemoprevention and/or the treatment of certain types of cancer in association with conventional anticancer therapies. Therefore, our results could be promising in order to develop adjuvant phytotherapeutics for cancer treatment.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jsps.2021.04.016.

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