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ORIGINAL ARTICLE

Cytotoxic and antioxidant properties of phenolic compounds from *Tagetes patula* flower

Muhammad Kashif[1,2], Samina Bano[2], Sabira Naqvi[2], Shaheen Faizi[2], Lubna[2], M. Ahmed Mesaik[1], Khawaja Shamsuddin Azeemi[3], and Ahsana Dar Farooq[1,2]

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Abstract

Context: *Tagetes patula* Linn. (Asteraceae) (French Marigold) flowers are used by local practitioners for cancer treatment; however, it lacks scientific justification.
Objective: Identification of bioactive compounds in *T. patula* flower for cytotoxic and growth inhibition in human cancer cell lines along with its antioxidant properties using chemical and cell based systems.
Materials and methods: The *T. patula* flower methanol extract, its seven fractions, and three phenolic compounds including methyl protocatechuate (**1**), patuletin (**2**), and patulitrin (**3**) were evaluated using sulforhodamine-B assay against HeLa, HT-144, NCI-H460, MCF-7, PC-3, and SF-268 human cancer cell lines. In parallel, antioxidant activity was evaluated using chemical ($DPPH^{\cdot}$, deoxyribose, and lipid peroxidation assays) and cell-based chemiluminescence systems (human neutrophils and mice macrophages).
Results: The methanol extract and ethyl acetate insoluble fraction exhibited cytotoxic and growth inhibitory effects against HeLa in which **2** exhibited highest cell growth inhibition (GI_{50}: 0.6 ± 0.1 μg/ml) and cytotoxicity (LC_{50}: 2.5 ± 0.1 μg/ml). It also scavenged $LOO^{\cdot}$ (IC_{50}: 6.5 ± 0.7 μg/ml) and $O_{2^-}^{\bullet}$ (IC_{50}: 27.5 ± 1.3 μg/ml) in chemical systems and human neutrophils, respectively. However, **1** preferably scavenged H_2O_2–Cl^- (IC_{50}: 0.5 ± 0.01 μg/ml) in mice macrophages.
Discussion and conclusion: Compound **2** from *T. patula* flower exhibited both growth inhibitory and cytotoxic properties while **1** and **3** were only growth inhibitory against HeLa. **1**–**3** also displayed antioxidant properties implying its probable role in growth inhibition/cytotoxic action. The present study provides scientific evidence for the use of *T. patula* flower in cancer treatment by traditional healer.

Keywords

Antiproliferative, free radical scavenger, French marigold, HeLa cell line, patuletin

History

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Introduction

Cancer is a leading cause of death and disability causing approximately 7.6 million deaths each year globally (Beaglehole et al., 2011) and is a major public health concern in many parts of the world. According to the World Health Organization (WHO) in 2009, about 750 million people died from cancer worldwide (Liu et al., 2012). Cervical cancer is the third most common cancer among women with 86% of global burden occurring in developing regions (De Sanjose et al., 2012). Although a number of chemotherapeutic agents are in clinical use against different types of cancers, however, undesirable side effects have been associated with them, e.g., cisplatin against cervical cancer (Zagouri et al., 2012) induces nephrotoxicity, paclitaxel exhibits musculoskeletal pain in breast and lung cancer patients (Heney et al., 2010), and vincristine used for leukemia is neurotoxic (Vilpo et al., 2000). The mechanism involved in cisplatin-induced nephrotoxicity is due to reduction of antioxidant status of kidney (Kilic et al., 2013) and vincristine neurotoxicity occurs via activation of glycogen synthase kinase-3 pathway by destabilization of microtubules that are abundant in nervous tissue (Alimoradi et al., 2012).

In spite of tremendous development in synthetic drugs, plants still constitute one of the major sources for modern drug development (Rahman et al., 2011). This is clearly reflected by many plant derived anticancer drugs, e.g., taxol from *Taxus brevifolia* Nuttall (Texaceae), vinblastine, and vincristine from *Catharanthus roseus* L. G. Don (Apocynaceae), and etoposide and teniposide from *Podophyllum* Linn (Berberidaceae) (Shoeb, 2006). The growing interest in plant natural products for anticancer activity is due to their secondary metabolites like terpenes,

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phenolics, and alkaloids (Dai & Mumper, 2010). There are many examples of plants rich in phenolic compounds that have demonstrated growth inhibitory and cytotoxic activities due to the presence of different types of flavonoids, e.g., quercetin, rutin, and apigenin in *Brassica oleracea italica* × *albograbla* against colon (SW480) and liver cancer (HepG2) cells (Wang & Zhang, 2011), quercetin, catechin, and fistein in *Zingiber officinale* against human breast cancer (MCF-7 and MDA-MB-231) (Rahman et al., 2011) and kaempferol, isorhamnetin, and rutin in *Chenopodium quinoa* against rat prostate cancer (AT-2) and human melanoma (HTB-140) cell lines (Dziki et al., 2013). The flavonoids quercetin, apigenin, and kaempferol have also demonstrated antitumor activity in the xenograft mouse model against human lung (Chan et al., 2013), colon (Shao et al., 2013), and osteosarcoma (Huang et al., 2010), respectively. The flavones flavopiridol and phenoxodiol are under clinical trials against prostate (Newcomb, 2004) and ovarian (Kelly et al., 2011) cancers. Most recently, a comprehensive review on Australian fruits (used as food and medicine by aboriginals) rich in phenolic compounds (apigenin, genestin, and gallic acid) demonstrated high antioxidant and antiproliferative properties associated with multiple mechanisms such as down regulation of NF-k B, pAKT, and inhibition of intrinsic mitochondrial pathway (Vuong et al., 2014). These phenolic compounds have been proposed as promising future anti-pancreatic cancer candidates emphasizing the folklore use of plants against cancer treatment.

Tagetes patula Linn. (Asteraceae) (French Marigold) is edible and used in folklore for the treatment of colics, diarrhea, vomiting, fever, skin, and hepatic diseases (Rondon et al., 2006). This plant possesses various biological properties including nematicidal (Faizi et al., 2011a), antibacterial, antifungal (Faizi et al., 2008), anti-inflammatory (Kasahara et al., 2002), analgesic, and antioxidant (Faizi et al., 2011b). Its phytochemical investigation revealed the presence of alkaloid (Faizi & Naz, 2002), flavonoids, thiophenes as well as terpenes (Vasudevan et al., 1997). A Pakistani scholar of international repute and traditional healer, Khawaja Shamsuddin Azeemi, is using *T. patula* flower petals in early morning under fasting condition for all types of cancer patients with promising results (Azeemi, 1999). Keeping this in mind, bioassay-directed cytotoxic and growth inhibitory activities of *T. patula* flower were investigated using human cancer cell lines and its antioxidant properties were evaluated using chemical and cellular antioxidant assays.

Materials and methods

Chemicals

1,1-Diphenyl-2-picryl-hydrazyl (DPPH), 2-deoxy-D-ribose, ascorbic acid, bovine brain extract Type VII, butylhydroxytoluene (BHT), dimethyl sulfoxide (DMSO), ethylene diaminetetraacetic acid (EDTA), ferric chloride ($FeCl_3$), ficoll paque (lymphocyte separation medium), fetal bovine serum (FBS), L-glutamine penicillin–streptomycin solution (GPSS), Hank's buffered saline solution (HBSS), hydrogen peroxide, lucigenin (*bis-N*-methyl-lacridinium nitrate), phorbol-12-myristate-13-acetate (PMA), potassium phosphate monobasic (KH_2PO_4), potassium phosphate dibasic ($K_2HPO_4 \cdot 3H_2O$), quercetin, Roswell Park Memorial Institute-1640 medium (RPMI-1640), sulforhodamine-B (SRB), rutin, thiobarbituric acid (TBA), trichloroacetic acid, trypsin-EDTA, and Tris base were obtained from Sigma (St. Louis, MO). Amphotericin-B (Forme Pharma, Karachi, Pakistan), gelatin (Scharlau, Barcelona, Spain), luminol (3-aminophthalhydrazide) (Alfa Aesar, Karlsruhe, Germany), and zymosan A (MP Biomedicals, Strasbourg, France) were purchased from respective companies.

Plant collection

Flowers of *T. patula* were collected during the months of September–January (2000–2003) from the University of Karachi campus. It was identified by Dr. Rubina Dawar of the Department of Botany, University of Karachi, Pakistan, and the voucher specimen (No. 67280) was deposited in the herbarium.

Extraction and isolation

Fresh, shade-dried, and uncrushed mix color flowers (5.2 kg) of *T. patula* were extracted three times with petroleum ether (PE) at room temperature (RT) (Figure 1). The PE extract was evaporated *in vacuo* into a gummy residue (JFP). The remaining marc-1 was extracted with methanol and evaporated under reduced pressure to obtain dried residue (JFM). This was first partitioned between distilled water (D/W) and PE to give PE phase (JFM-P) followed by extraction of aqueous phase with dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and butanol (BuOH). After a few days, insoluble matter deposited in the organic phases: JFM-P, JFM-DC, and JFM-EA (Faizi et al., 2008, 2011b).

The PE phase (JFM-P) was concentrated and decanted to obtain insoluble material (JFM-PI) and the decantate JFM-PD. The dichloromethane (CH_2Cl_2) phase was treated in the same way yielding the insoluble material (JFM-DC-I) and decantate soluble material (JFM-DC-D). Furthermore, needle-like crystals appeared in CH_2Cl_2 phases (JFM-DC-I), which were collected with spatula and dissolved in $CHCl_3$:MeOH (1:1). The needle-like white crystals reappeared which were filtered to give 70.3 mg of cyclooctasulfur S_8 (TLC = RP-18, MeOH, $R_f = 0.37$, pink spot under UV-254). The ethyl acetate phases afforded yellow flakes on concentration at RT that were also decanted furnishing the insoluble material and decantate (JFM-EA-D). The JFM-EA-D was further concentrated at RT and filtered to give filtrate JFM-EA-DF and insoluble matter JFM-EA-DI. The methanol yellowish filtrate (JFM-EA-DF) on concentration provided yellow powdery matter (1.7 g) which was filtered and washed several times with PE and CH_2Cl_2 to furnish patuletin (**2**) (TLC = $R_f = 0.28$, $CHCl_3$:MeOH 9:1). From the CH_2Cl_2 washing, methyl protocatechuate (**1**) was isolated as white crystals through preparative thin-layer chromatography (PTLC) (silica gel, $CHCl_3$:MeOH, 9.5:0.5) (Faizi et al., 2008, 2011b). All the butanol phases were combined together and evaporated *in vacuo* to give a residue, JFM-Bu. A small fraction of JFM-Bu was treated with methanol and then filtered to give 0.1 g of patulitrin (**3**) (Figure 2) (Faizi et al., 2008, 2011b).

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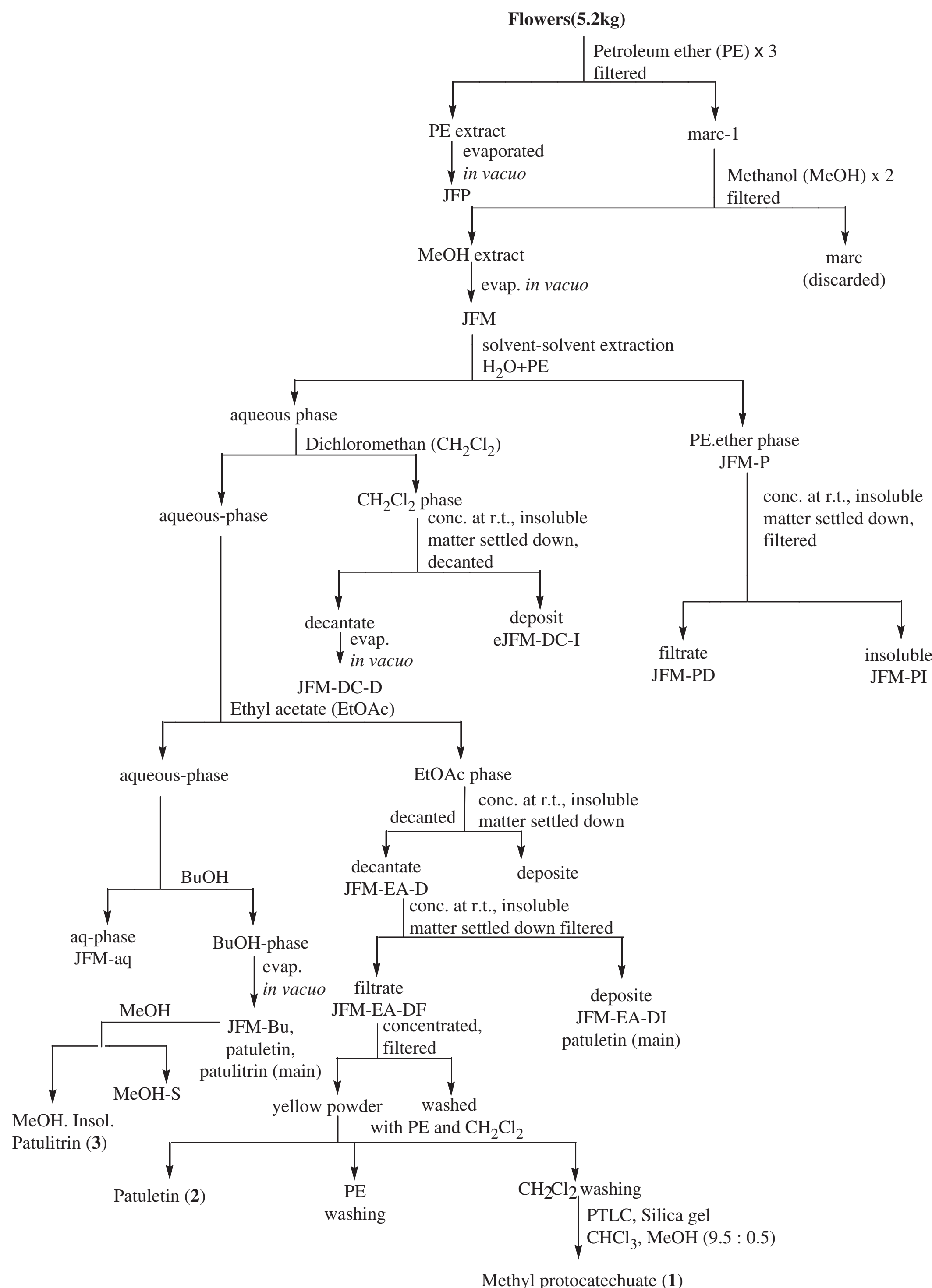

Figure 1. Bioassay-guided extraction and fractionation of *T. patula* flower.

Identification of compounds

Compounds **1** (methyl protocatechuate), **2** (patuletin), and **3** (patulitrin) were identified and compared with chemical/physical data reported by NMR and mass spectrometry in the literature (Faizi et al., 2010, 2011b; Valant-Vetschera et al., 2003).

Preparation of samples

The stock solutions of plant extract (JFM), fractions (JFM-P, JFM-PI, JFM-DC-D, JFM-DC-I, JFM-EA-DF, JFM-EA-DI, and JFM-Bu) (40 mg/ml) and compounds **1**, **2**, and **3** and cisplatin (20 mM) were prepared in DMSO (100%) with a final concentration of DMSO not exceeding 0.5% (v/v) while doxorubicin (20 mM) in sterile (D/W). Further dilutions were prepared in RPMI-1640 medium. For antioxidant assays, stock solutions (1 mg/ml) of samples including extracts/fractions/pure compounds were prepared in methanol (100%). The samples were diluted in ethanol for DPPH assay and phosphate buffer (10 mM, $KH_2PO_4 + K_2HPO_4$, pH 7.4) for deoxy ribose and lipid peroxidation, and HBSS buffer was used in chemiluminescence assay.

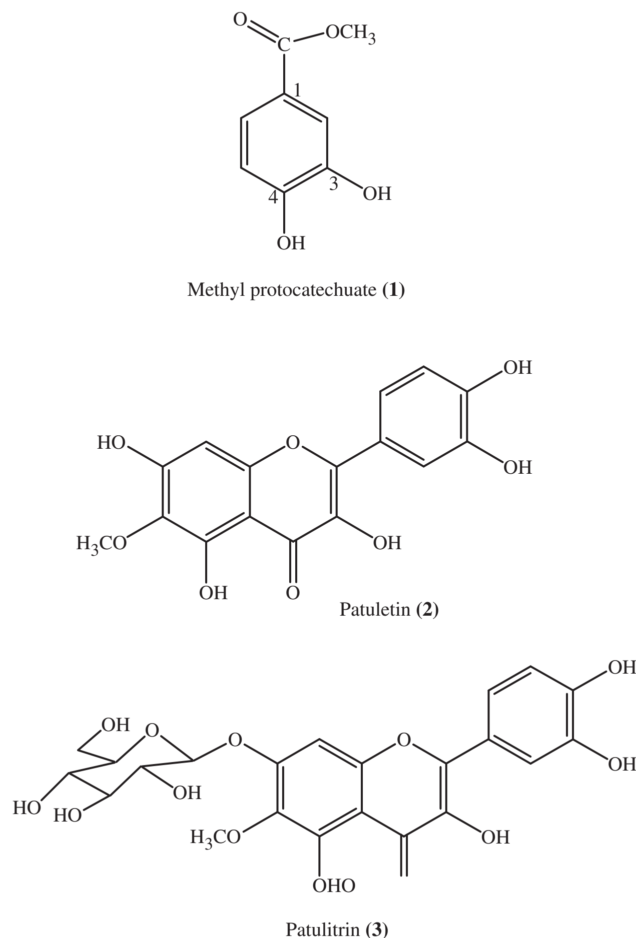

Figure 2. Chemical structure of pure compounds of *T. patula* flower.

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Human cancer cell lines

Human cancer cell lines, NCI-H460 (large cell carcinoma, lung) MCF-7 (adenocarcinoma, breast), and SF-268 (anaplastic astrocytoma, CNS) were kindly provided by the National Cancer Institute (NCI), Frederick, MD. The HT-144 (melanoma, skin) was obtained from Biomedical and Genetic Engineering Division, KRL, Islamabad, Pakistan, while HeLa (uterine cervix) and PC-3 (prostate) were obtained from ATCC (American Type Culture Collection). The cancer cell lines were maintained in culture medium (15 ml) containing serum (FBS, 10% v/v), L-glutamine (1% w/v or 200 mM), penicillin (1% w/v, 104 U/ml), streptomycin (100 μg/ml), and antifungal amphotericin-B (10 μg/ml) in culture flasks (75 ml). These were kept in the CO_2 incubator (5% CO_2) at 37 °C till a confluent monolayer was formed within 3–4 d.

Bioassay

SRB assay

The *in vitro* cytotoxicity assay used was initially developed at NCI in 1990 for pre-clinical screening of natural products and synthetic compounds against a panel of 60 different human cancer cell lines. It is still most widely used assay as it provides information about both cell growth inhibition and cytotoxicity (Qamar et al., 2010; Skehan et al., 1990). The monolayer was trypsinized for 2–3 min with trypsin/EDTA (0.05%/0.02%) to obtain cell suspension. The cell concentration and viability were determined for each cell line using a hemocytometer. Cell suspension (100 μl/well) with appropriate cell number of HT-144 (2×10^4), MCF-7, PC-3, HeLa, NCI-H460 (1×10^4), and SF-268 (1.5×10^4) was added in each well of 96-well plates except the blank and incubated at 37 °C in 5% CO_2 for 24 h. Test agents (100 μl) including *T. patula* extract (1–250 μg/ml), fractions (1–100 μg/ml), and pure compounds **1–3** (0.001–100 μM) along with anticancer drugs doxorubicin (0.001–10 μM) and cisplatin (0.1–100 μM) were added in the appropriate wells. The plates were then incubated for 48 h followed by fixation with ice-cold trichloroacetic acid (50 μl, 50%) for 30 min and washed with distilled water. The plates were air-dried overnight and stained with SRB (0.4%) for 30 min followed by the removal of unbound stain with acetic acid (1%). Tris-base (100 μl, 10 mM) was used to solubilize SRB stain and optical density was recorded spectrophotometrically at 515 nm. The GI_{50} (50% growth inhibition) and LC_{50} (50% cell death) indicating the sample concentration for 50% growth inhibition and cell death of total cells, respectively, were obtained graphically as concentration versus percent cell growth inhibition and cytotoxicity.

DPPH free radical-scavenging assay

The DPPH assay was conducted as described by Mensor et al. (2001). The test agents (1.5 ml) including *T. patula* extract, fractions (2.5–100 μg/ml), pure compounds (2–50 μg/ml), and standard antioxidants, namely quercetin and rutin (2–20 μg/ml) were added in ethanolic DPPH solution (1 ml, 300 μM) in a total volume of 2.5 ml. After 30 min of incubation at RT, absorbance was recorded at 518 nm using a spectrophotometer (UV–vis spectrophotometer, Secomam, Alès Cedex, France). The IC_{50} values (concentration causing 50% DPPH radical-scavenging activity) were obtained graphically between the concentration versus percent free radical-scavenging activity.

Deoxyribose degradation assay

The assay was performed in a reaction mixture (500 μl) containing 250 μl of test agents (1–50 μg/ml), 2-deoxy-D-ribose (50 μl, 28 mM), EDTA and $FeCl_3$ (1:1, 100 μl, 1 mM), and H_2O_2 and ascorbic acid each (50 μl, 1 mM). The reagents were mixed gently and incubated at 37 °C for 1 h. The control reaction contained equal volume of phosphate buffer instead of test agents. TBA (500 μl, 1% in 50 mM NaOH) and trichloroacetic acid (500 μl, 2.8 % in D/W) were added and the mixture was heated at 100 °C for 20 min. Upon cooling, the absorbance of pink chromogens generated was measured at 532 nm against a blank containing deoxyribose and phosphate buffer (Halliwell et al., 1987).

Lipid peroxidation assay

The test agents (150 μl, 1–100 μg/ml) were reacted with bovine brain extract (250 μl, 0.5 mg/ml), $FeCl_3$, and ascorbic acid each (50 μl, 1 mM), whereas control contains equal volume of phosphate buffer in the place of test agents. After incubation at 37 °C, TBA, TCA, and butylhydroxytoulene (50 μl, 1% in methanol) were added and heated at 100 °C for

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20 min followed by the addition of *n*-butanol (1.25 ml). The reaction mixture was centrifuged at 3000 rpm and an organic layer (top layer) was aspirated. The absorbance of this top layer was measured at 532 nm against a blank containing bovine brain phospholipids and phosphate buffer (Houghton et al., 1995).

Chemiluminescence assay

The scavenging activity of reactive oxygen species (ROS) generated intracellularly or extracellularly was measured using luminol and lucigenin chemiluminescence probes, respectively, as described by Helfand et al. (1982). The test agents (25 µl, 0.5–100 µg/ml) were incubated at 37 °C either with human whole blood (25 µl, 1:20 dilution in HBSS) or human neutrophils or mice macrophages (25 µl, 1×10^6 cells/ml) for 15 min in a 96-well plate. The opsonized zymosan (25 µl, 3 mg/ml) or phorbol-myristate-acetate (25 µl, 1.3×10^{-7} M) was added as an intra- or extracellular stimulator of ROS, respectively. After addition of luminol (25 µl, 1.53 mM) or lucigenin (25 µl, 5 mM), chemiluminescence was measured on repeated scan mode nm in a luminescence reader as relative light units (RLUs).

Statistical analysis

One-way analysis of ANOVA followed by Duncan multiple comparisons was used to calculate differences between the control and treated groups. The p value <0.05*, <0.01**, and <0.001*** were considered significant.

Results

The methanol extract (JFM) and its fractions (JFM-P, JFM-PI, JFM-DC-D, JFM-DC-I, JFM-EA-DF, JFM-EA-DI, and JFM-Bu) along with three pure compounds, namely methyl protocatechuate (**1**), patuletin (**2**) and patulitrin (**3**) of *T. patula* flower were evaluated for growth inhibitory, cytotoxic, and antioxidant activities as described below.

Growth inhibitory and cytotoxic activity

The methanol extract (1–250 µg/ml) and its fractions (1–100 µg/ml) demonstrated growth inhibitory activity against all the human cancer cell lines used (Figure 3). However, the methanol extract was most effective against HeLa cells showing both growth inhibitory (GI_{50}: 7.3 ± 0.9 µg/ml) and cytotoxic properties (LC_{50}: 37 ± 0.3 µg/ml) (Table 1). Among fractions, the ethyl acetate insoluble fraction (JFM-EA-DI) was consistently more effective against HeLa cell line (GI_{50}: 3.5 ± 0.3 µg/ml and LC_{50}: 25 ± 1.1 µg/ml). Its further fractionation yielded methyl protocatechuate (**1**) and two flavonoids, namely patuletin (**2**) and patulitrin (**3**). Among them, compound **2** was the most active exhibiting growth inhibitory as well as cytotoxic properties (GI_{50}: 0.6 ± 0.1 µg/ml and LC_{50}: 2.5 ± 0.1 µg/ml), whereas compound **1** (GI_{50}: 9.3 ± 0.1 µg/ml) and **3** (GI_{50}: 5 ± 2 µg/ml) demonstrated only growth inhibitory potential. The growth inhibition of doxorubicin and cisplatin (GI_{50}: 0.35 ± 0.02 µg/ml and 1.2 ± 0.24 µg/ml) and their respective cytotoxicities (LC_{50}: 4 ± 0.08 µg/ml and 15 ± 0.6 µg/ml) against HeLa cell line were also evaluated. The GI_{50} potency order appears to be doxorubicin = patuletin (**2**) = cisplatin > JFM-EA-DI > patulitrin (**3**) > JFM > methyl protocatechuate (**1**), whereas the potency order for LC_{50} was patuletin (**2**) > doxorubicin > cisplatin > JFM-EA-DI > JFM > patulitrin (**3**) = methyl protocatechuate (**1**).

Antioxidant activity

Free radical-scavenging activity was evaluated using (i) chemical assays including DPPH, deoxyribose degradation, and lipid peroxidation to scavenge 1,1-diphenyl-2-picrylhydrazyl ($DPPH^\bullet$), hydroxyl ($OH^\bullet$), and peroxy radicals ($LOO^\bullet$) respectively, whereas (ii) cell-based chemiluminescence assay was applied using human whole blood, neutrophils, and mice macrophages for scavenging ROS including superoxide anion ($O_{2^-}^{\bullet}$) and hydrogen peroxide–hypochlorite $(H_2O_2–Cl)^-$.

Chemical-based free radical-scavenging activity

Against $DPPH^\bullet$ radical, the methanol extract of *T. patula* flower exhibited IC_{50}: 41 ± 0.8 µg/ml while its ethyl acetate insoluble fraction was about seven-times (~7×) more potent (Table 2). Among pure compounds, methyl protocatechuate (**1**) (IC_{50}: 2.1 ± 0.06 µg/ml) showed two-times (2×) and three-times (3×) more potent activity than patuletin (**2**) and patulitrin (**3**), respectively. In contrast, in the deoxyribose assay, the methanol extract (IC_{50}: 5.8 ± 1.1 µg/ml) was more active than its ethyl acetate insoluble fraction (1.5×) in scavenging $OH^\bullet$ while in the lipid peroxidation assay, again ethyl acetate insoluble fraction was 3× more potent than the methanol extract (IC_{50}: 23 ± 3 µg/ml). Compound **1** was the most active scavenger of $OH^\bullet$ (IC_{50}: 2.75 ± 0.5 µg/ml) which exhibited ~3× and ~6× more potent effects than that of compounds **2** and **3**, respectively. However, against $LOO^\bullet$ radical, compound **2** (IC_{50}: 6.5 ± 0.7 µg/ml) was ~5× and ~3× more active than **1** and **3**, respectively (Table 2). Thus, potency order to scavenge $DPPH^\bullet$ and $OH^\bullet$ appears to be compound **1** >compound **2** > compound **3**; whereas, in case of $LOO^\bullet$ radical, the potency order appears to be compound **2** >compound **3** >compound **1**.

Cell-based (ROS)-scavenging activity

The methanol extract showed a similar potency (IC_{50}: ~30 µg/ml) in scavenging superoxide anion ($O_{2^-}^{\bullet}$) in human neutrophils and hydrogen peroxide–hypochlorite ($H_2O_2–Cl^-$) in mice macrophages whereas its ethyl acetate insoluble fraction was 2× more active against $H_2O_2–Cl^-$ but ineffective against $O_{2^-}^{\bullet}$ (Table 3). Among the pure compounds, methyl protocatechuate (**1**) was the most potent in scavenging $H_2O_2–Cl^-$ (IC_{50}: 0.5 ± 0.01 µg/ml) in mouse macrophages and was 6× and 32× more potent than compounds **2** and **3**, respectively (Table 3). Its activity to scavenge $H_2O_2–Cl^-$ in mouse macrophages was ~8× more potent than those of widely used antioxidants, quercetin and rutin. In human neutrophils, patuletin (**2**) scavenged $O_{2^-}^{\bullet}$ (IC_{50}: 27.5 ± 1.3 µg/ml) more actively than compound **1** (1.4×) while compound **3** was ineffective against superoxide ($O_2^{\cdot-}$) radical.

Discussion

The *Tagetes* species have been reported earlier to possess cytotoxic activity as aqueous and ethanol extracts from aerial

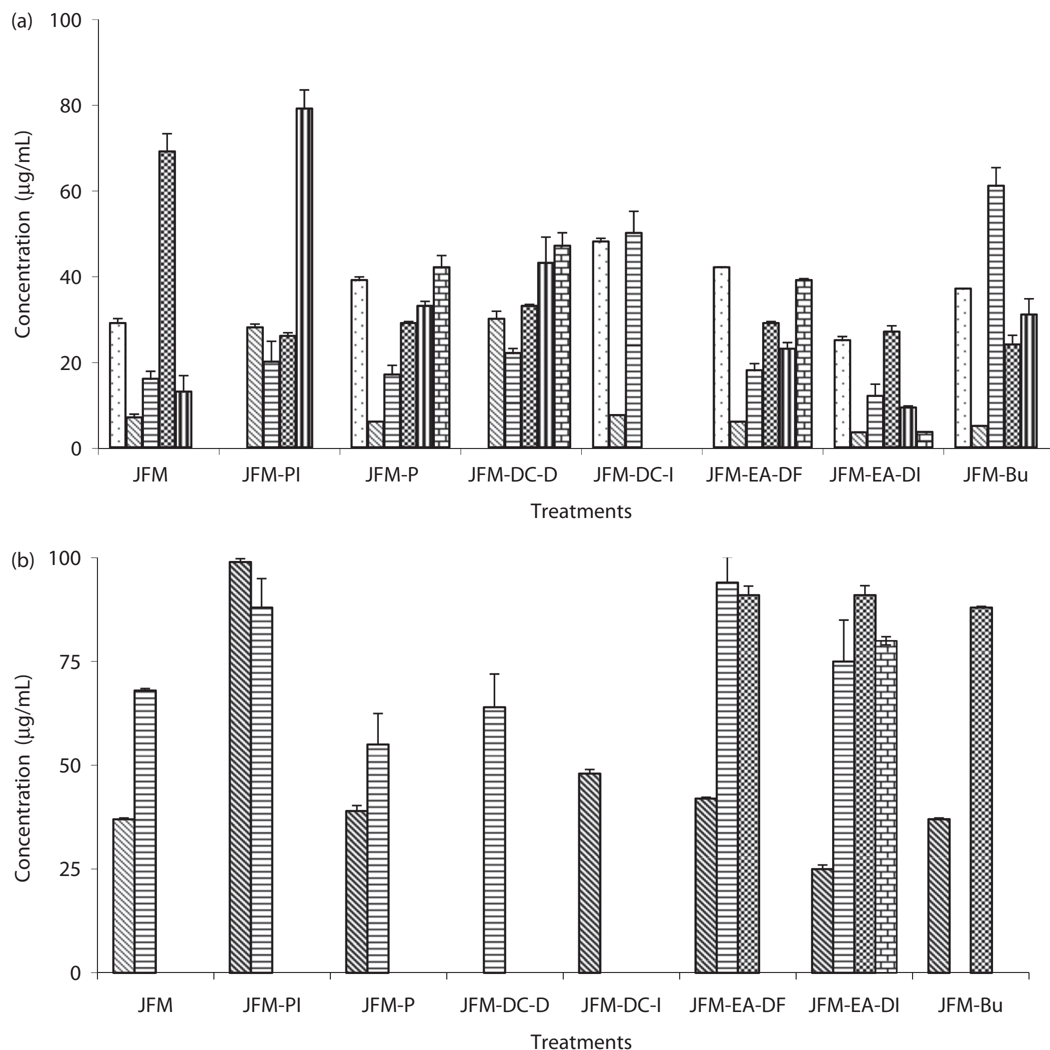

Figure 3. The bar graph represents concentrations values of (a) GI_{50} (50% cell growth inhibition) and (b) LC_{50} (50% cell kill) of *T. patula* methanol extract (JFM) and its fractions (JFM-PI, JFM-P, JFM-DC-D, JFM-DC-I, JFM-EA-DF, JFM-EA-DI, and JFM-Bu) against PC-3 (), HeLa (), HT-144 (), MCF-7 (), SF-268 (), and NCI-H460 () cancer cell lines.

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parts of *T. lucida* were effective against HeLa ($GI_{50:}$ 13.2 µg/ml and GI_{50}: >50 µg/ml) and breast cancer (T47D) cell lines ($GI_{50:}$ 18.9 µg/ml and $GI_{50:}$ 1.82 µg/ml), respectively (Vega-Avila et al., 2009). Similarly, aqueous extract of *T. minuta* was effective against Lewis lung carcinoma of mouse C57BL (43% treated/control value at 266 mg/kg) (Ickes et al., 1973). In the present study, methanol extract of *T. patula* flower also exhibited growth inhibition and cytotoxicity against various human cancer cell lines including HT-144, HeLa, MCF-7, NCI-H460, SF-268, and PC-3; however, it was the most active (GI_{50}: 7.3 ± 0.9 µg/ml and LC_{50}: 37 ± 0.3 µg/ml) against HeLa as reflected by lowest GI_{50} and LC_{50} values suggesting *Tagetes* species sensitivity towards it. In HeLa cell line, bioassay-guided fractionation of methanol extract of *T. patula* flower afforded ethyl acetate-insoluble fraction that exhibited ~2-fold better activity, whereas patuletin isolated from the same fraction showed profound growth inhibitory (GI_{50}: 12× and 6×) and cytotoxic (LC_{50}: 15× and 10×) effects compared with extract and fraction, respectively. This improvement of bioassay-guided growth inhibitory, and cytotoxic effects of *T. patula* methanol extract and ethyl acetate fraction are more likely due to the presence of phenolic compounds (flavonoids and phenolic acid) that prefer solvents of medium polarity (Hincapie et al., 2011). The ethyl acetate insoluble fraction of *T. patula* flower on purification also revealed major flavonoid, patuletin (**2**), and its 7-*O*-glucoside, patulitrin (**3**), along with an ester, methyl protocatechuate (**1**) (Faizi et al., 2008, 2010). The ethyl acetate fractions from other *Tagetes* spp., such as *T. maxima* (Parejo et al., 2005) and *T. erecta* (Valyova et al., 2012) were also reported to be flavonoid rich with high phenolic contents. There are also many examples of plants with polyphenol-rich methanol extracts such as *Mangifera indica* (Joona et al., 2013), *Ipomoea pes-caprae* (Parekh et al., 2012), and *Ficus elastica* Roxb exhibiting cytotoxic properties (EL-Hawary et al., 2012).

The comparison of patuletin with two clinically used drugs, doxorubicin and cisplatin, indicated that it was (2-fold) better growth inhibitor than cisplatin, a drug of choice in cervical cancer treatment (Zagouri et al., 2012) implying that patuletin is a promising cytotoxic molecule against cervical cancer derived from plants. In contrast, it was (1.6-fold) more cytotoxic than doxorubicin which is used against breast cancer (Prados et al., 2012). The cytotoxic studies of patuletin against normal human fetal lung fibroblast (MRC-5) cell line

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Table 1. Growth inhibitory and cytotoxic effects of extract, fraction and pure compounds of *T. patula* flower and standard anticancer agents against HeLa cell line.

Treatment	Doses (μg/mL)	% Cell growth inhibition/kill	(μg/mL) GI_{50}	LC_{50}
Methanol extract (JFM)	1	$+15 \pm 5.00$	7.30 ± 0.90^{d}	37.00 ± 0.30^{e}
	10	$+62 \pm 2.00$***		
	50	-91 ± 0.60***		
	100	-97 ± 1.00***		
	250	-98 ± 1.00***		
Ethyl acetate insoluble fraction (JFM-EA-DI)	1	$+15 \pm 1.00$	3.50 ± 0.30^{b}	25.00 ± 1.10^{d}
	5	$+87 \pm 2.00$***		
	10	-31 ± 1.00***		
	50	-80 ± 2.00***		
	100	-94 ± 2.00***		
Pure compounds	(μM)			
Patuletin	0.001	$+1.0 \pm 0.10$	0.60 ± 0.10^{a} (2.00 ± 0.10)	2.50 ± 0.10^{a} (7.50 ± 0.20)
	0.01	$+1.0 \pm 0.10$		
	0.1	$+2.0 \pm 0.50$		
	1	$+35 \pm 3.00$**		
	10	-91 ± 5.00***		
Patulitrin	0.1	$+17 \pm 6.00$	5.00 ± 2.00^{c} (11.00 ± 3.00)	>100
	1	$+32 \pm 9.00$		
	5	$+35 \pm 8.00$		
	50	-22 ± 4.00		
	100	-48 ± 4.00***		
Methyl protocatechuate (HBAM)	0.01	$+2.0 \pm 1.00$	9.30 ± 0.10^{e} (57.00 ± 0.60)	>100
	0.1	$+2.0 \pm 1.00$		
	1	$+6.0 \pm 2.00$		
	10	$+8.0 \pm 1.00$*		
	100	$+95 \pm 0.60$***		
Anticancer drugs				
Doxorubicin	0.001	$+4.0 \pm 0.40$	0.35 ± 0.02^{a} (0.60 ± 0.03)	4.00 ± 0.08^{b} (7.10 ± 0.10)
	0.01	$+5.0 \pm 1.00$		
	0.1	$+26 \pm 2.00$**		
	1	$+69 \pm 3.00$***		
	10	-94 ± 1.00***		
Cisplatin	0.1	$+25 \pm 2.00$**	1.20 ± 0.24^{a} (4.00 ± 0.80)	15.00 ± 0.60^{c} (46.00 ± 2.00)
	1	$+42 \pm 2.00$***		
	10	$+66 \pm 1.00$***		
	50	-69 ± 8.00***		
	100	-63 ± 8.00***		

Control absorbance (520 nm): (2.2 ± 0.3). Growth inhibition: +; cytotoxicity: −. Each value represents percent mean ± SEM as compared to control ($n = 3$). GI_{50} and LC_{50} are concentrations of drug causing 50% cell growth inhibition and kill. Asterisk indicates significant ($p < 0.05$*, $p < 0.01$** and $p < 0.001$***) growth inhibition and/or cytotoxicity compared with the respective controls. Values within parenthesis are expressed in (μM).
$^{a–e}$Homogeneous means are represented by similar alphabetical superscripts in vertical columns.

Table 2. IC_{50} values representing DPPH•, OH•, and LOO• radicals scavenging activity of extract, fraction, and pure compounds of *T. patula* flower and standard antioxidant agents.

Treatment	(μg/mL) DPPH assay (DPPH•)	Deoxy ribose assay (OH•)	Lipid peroxidation assay (LOO•)
Methanol extract (JFM)	$41.00 \pm 0.80^{d,2}$	$5.80 \pm 1.10^{a,b,1}$	$23.00 \pm 3.00^{c,2}$
Ethyl acetate insoluble fraction (JFM-EA-DI)	$6.50 \pm 0.2^{c,1}$	$9.00 \pm 1.50^{b,1}$	$8.60 \pm 0.50^{a,1}$
Pure compounds			
Methyl protocatechuate (HBAM)	$2.10 \pm 0.06^{a,1}$	$2.75 \pm 0.50^{a,1}$	$30.00 \pm 2.60^{d,2}$
Patuletin	$3.80 \pm 0.2^{b,1}$	$7.80 \pm 1.00^{b,2}$	$6.50 \pm 0.70^{a,1}$
Patulitrin	$6.40 \pm 0.12^{c,1}$	$17.00 \pm 1.00^{c,2}$	$17.00 \pm 1.70^{b,2}$
Standard agents			
Quercetin	$3.30 \pm 0.25^{b,1}$	$6.20 \pm 1.00^{a,b,2}$	$6.90 \pm 0.50^{a,2}$
Rutin	$7.33 \pm 0.10^{c,1}$	$7.30 \pm 1.00^{b,1}$	$>100^{e,2}$

Each value represents percent mean ± SEM of three independent experiments conducted in triplicate. IC_{50} indicates concentration of drug causing 50% scavenging of DPPH•, hydroxyl (OH•), and peroxy (LOO•) radicals.
$^{a–d}$Homogenous means are represented by similar alphabetical superscripts in columns.
$^{1–2}$Homogenous means are represented by similar numerical superscripts in rows.

Table 3. IC_{50} values representing H_2O_2–Cl^- and $O_{2^-}^{\bullet}$ radicals scavenging effects of extract, fraction, and pure compounds of *T. patula* flower along with standard antioxidant agents.

Treatment	Human whole blood (H_2O_2–Cl^-) (μg/mL)	Human neutrophils (H_2O_2–Cl^-) (μg/mL)	Human neutrophils ($O_{2^-}^{\bullet}$) (μg/mL)	Mice macrophages (H_2O_2–Cl^-) (μg/mL)
Methanol extract (JFM)	$56.00 \pm 0.30^{d,3}$	$39.60 \pm 2.40^{c,2}$	$30.60 \pm 1.60^{b,1}$	$28.60 \pm 0.300^{d,1}$
Ethyl acetate insoluble fraction (JFM-EA-DI)	$14.00 \pm 2.00^{b,1}$	$33.00 \pm 0.30^{b,2}$	$>50.00^{d,2}$	$14.30 \pm 3.00^{c,1}$
Pure compounds				
Methyl protocatechuate (HBAM)	$3.00 \pm 0.36^{a,2}$	$2.75 \pm 0.200^{a,2}$	$38.00 \pm 1.30^{c,3}$	$0.50 \pm 0.01^{a,1}$
Patuletin	$3.50 \pm 0.20^{a,1}$	$3.40 \pm 0.20^{a,1}$	$27.50 \pm 1.30^{a,2}$	$3.00 \pm 0.10^{b,1}$
Patulitrin	$6.40 \pm 1.30^{b,1}$	$32.00 \pm 2.50^{b,2}$	$>50.00^{d,3}$	$16.30 \pm 1.30^{c,1}$
Standard agents				
Quercetin	$7.00 \pm 0.30^{a,b,2}$	$3.90 \pm 0.20^{a,1}$	$>50.00^{d,3}$	$3.30 \pm 0.80^{b,1}$
Rutin	$23.00 \pm 3.60^{c,2}$	$29.60 \pm 2.80^{b,2}$	$>50.00^{d,4}$	$4.50 \pm 0.10^{b,1}$

Each value represents percent mean ± SEM of three independent experiments conducted in triplicate. IC_{50} indicates concentration of drug causing 50% scavenging of H_2O_2–Cl^- and superoxide ($O_{2^-}^{\bullet}$) radicals.
[a–d]Homogenous means are represented by similar alphabetical superscripts in columns.
[1,4]Homogenous means are represented by similar numerical superscripts in rows.

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(GI_{50}: 171 μg/ml) (Schmeda-Hirschmann et al., 2004) and intraperitoneal administration (100 mg/kg) in NMRI mice did not exhibit any mortality, also proven its safety (Faizi et al., 2011b) towards normal cells.

Patuletin shows certain features of flavonoids such as orthocatechol moiety in the B ring, unsaturated C2–C3 double bond, and hydroxylation at C-3 position that are crucial for flavonoid's antiproliferative action (Kawaii et al., 1999). Additionally, the presence of a methoxy group at its C-6 position further increases its cytotoxic effect (Yanez et al., 2004) as methylation of hydroxyl groups in flavonoids improves lipophilicity (Heim et al., 2003) due to their enhanced entry into cells (Fiuza et al., 2004). A similar phenomenon has also been observed for quercetagetin (Qt), a characteristic flavonoid of genus *Tagetes* (Abdala, 2003) and a parent molecule of patuletin (6-methyl ether quercetagetin) lacking the methyl group which exhibited very weak antiproliferative activity against HeLa cell line (GI_{50}: 168 μM) (Mori et al., 1988), while its polymethoxylated derivatives, centaureidin (trimethyl ether Qt, GI_{50}: 0.082 μM) and casticin (tetramethyl ether Qt, $GI_{50:}$ 1.28 μM), showed better activities (Csupor-Loffler et al., 2009). Recently, polymethoxyflavone (naringenin) was found to be more cytotoxic as compared with their non-methylated analogues (Wesolowska et al., 2012). In our study, both patulitrin (**3**, a glucosidic patuletin) and methyl protocatechuate (**1**, phenolic acid with methyl ester) exhibited weak antiproliferative activity as compared with patuletin (**2**). The lower activity of **3** is possible due to *O*-glycosylation (replacement of OH moiety with glucose at C-7 position of patuletin) (Cao et al., 1997); while in the presence of methyl protocatechuate (**1**), this might be related to the shorter alkyl length (Fiuza et al., 2004) leading to reduced lipophilicity (Masaki et al., 1997).

It is widely accepted that cancer cells are under oxidative stress (Kobayashi & Suda, 2012) displaying higher ratios of $O_{2^-}^{\bullet} \div H_2O_2$ in human breast ($2 \div 20\times$) and colon cancer cells ($1.8 \div 10\times$) as compared with their normal cells (Aykin-Burns et al., 2009). H_2O_2 activates mitogen-activated protein kinase (MAPK) signaling pathway (Muller et al., 1997) and activator protein-1 (AP-1) transcription factor (Loo, 2003) which ultimately triggers cell proliferation (Reuter et al., 2010). These free radicals and ROS are neutralized or scavenged by phenolic compounds, thereby preventing the growth of cancer cells (Vuong et al., 2014). Also, plants rich in flavonoids have been reported to possess both antioxidant and cytotoxic activities (Prasad et al., 2009). Therefore, *T. patula* flower was evaluated for antioxidant properties against ROS.

The methanol extract and phenolic compounds **1** and **2** of *T. patula* flower attenuated the production of luminol and lucigenin-amplified chemiluminescence against human neutrophils and mice macrophages, respectively, which is dependent on the production of H_2O_2 and O_2^-, respectively (Edwards, 1996; Dahlgren & Karlsson, 1999; Wymann et al., 1987). Also, in lucigenin-amplified chemiluminescence, neutrophils and macrophages are stimulated through protein kinase C (PKC) activation which is a cellular receptor for tumor-promoting phorbol ester (Blumberg, 1988) and produces mitogenic effects in early G1 cell cycle (Livneh & Fishman 1997) leading to cell proliferation (Nishizuka, 1992). In addition to cellular antioxidant effects, methyl protocatechuate (**1**) also actively scavenged $DPPH^{\cdot}$ (IC_{50}: 2.1 ± 0.06 μg/ml) and hydroxyl radical (IC_{50}: ~ 3 μg/ml), whereas patuletin (**2**) emerged as a potent scavenger of peroxy radical (IC_{50}: 6.50 ± 0.7 μg/ml) in the chemical antioxidant system. This is in agreement with earlier report where ester of protocatechuic acid acted as the potent scavenger of hydroxyl ion (Yen et al., 2005); whereas patuletin scavenged peroxy radical ($LOO^{\cdot}$) possibly due to the presence of dihydroxy group at carbon 3′ and 4′ positions (catechol moiety), a property of most flavonoids (Cao et al., 1997).

The strong antioxidant properties of *T. patula* flower indicates that its antiproliferative and cytotoxic properties might be related to antioxidant potential of its phenolic compounds specially flavonoid patuletin. However, other mechanisms associated with flavonoid induced cytotoxicity such as cell-cycle arrest (Yanez et al., 2004), topoisomerase, and enzyme kinase inhibition (Bandele et al., 2008; Kanadaswami et al., 2005) cannot be ignored and evaluation

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of patuletin addressing them will allow better understanding of its cytotoxic mechanism of action.

Conclusion

The bioassay-guided activity of a methanol extract of *T. patula* flower led to the isolation of patuletin (**2**), a principal flavonoid, mainly responsible for its cytotoxic and growth inhibitory activities preferably towards cervical cancer (HeLa cell line). Its glucoside, patulitrin (**3**) and phenolic acid, methyl protocatechuate (**1**) also exhibited growth inhibition. One possible mechanism of their growth inhibitory or cytotoxic activities appears to be related with their antioxidant properties. Thus, the present study justifies the folkloric use of *T. patula* flower against cancer treatment.

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Declaration of interest

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