Clinical Phytoscience

ORIGINAL CONTRIBUTION Open Access

Anti-proliferative and immunomodulatory activities of fractions from methanol root extract of *Abrus precatorius* L

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Abstract

Background: *Abrus precatorius* possesses various therapeutic properties including anticancer potentials. This study evaluated the anti-proliferative activities of fractions of methanol root extract of *A. precatorius* on breast and cervical cancer cells and their immunomodulatory effect. Phytochemical screening was done by FTIR and GCMS. In vitro anti-proliferative effect was evaluated on human breast cancer (AU565) and cervical cancer (HeLa) cells and on murine fibroblast (NIH 3 T3) cells. Antioxidant activity was performed via DPPH radical scavenging assay. The immunomodulatory potential of fractions was evaluated by inhibition of phagocytes oxidative burst (ROS), Nitric oxide (NO) and proinflammatory cytokine TNF-α.

Results: *A. precatorius* fractions showed different chemical groups and were somewhat selective in antiproliferative activity against studied cancer cells. Ethyl acetate fraction showed the most significant antiproliferative activity with IC_{50} values of 18.10 μg/mL and 11.89 μg/mL against AU565 and HeLa cells respectively. Hexane fraction significantly ($p < 0.05$) inhibited HeLa cells (IC_{50} 18.24 ± 0.16 μg/mL), whereas aqueous fraction showed mild inhibition (IC_{50} 46.46 ± 0.14 μg/mL) on AU565 cell proliferation. All fractions showed no cytotoxicity against NIH-3 T3 murine fibroblast normal cells. All fractions showed potent and significant ($p < 0.001$) DPPH radical scavenging activity as well as suppressed phagocytic oxidative burst. Hexane (< 1 μg/mL), ethyl acetate (< 1 μg/mL), and butanol (5.74 μg/mL) fractions potently inhibited the cytokine TNF- α, hexane (< 1 μg/mL) and ethyl acetate (< 1 μg/mL) fractions also potently inhibited NO.

Conclusions: The antiproliferative activities and suppressive effect on the phagocytic oxidative burst, NO and proinflammatory cytokine might be due to the synergistic actions of bioactive compounds especially flavonoids present in the assayed fractions and therefore, suggest chemotherapeutic use of *A. precatorius* in cancer treatment.

Keywords: *Abrus precatorius*, Breast cancer, Cervical cancer, Cytokines, Cytotoxicity, Tumor necrosis factor, oxidative burst

Introduction

Cancer is one of the leading causes of death in both developed and underdeveloped countries. It has generated considerable scientific and commercial interest especially in the progressive discovery of new anticancer agents from natural product sources. Cancer is broadly described as a group of diseases characterized by uncontrolled growth and spread of abnormal cells, associated with resistance to normal growth-inhibitory signals, uncontrolled activation of growth signals, impairment of apoptosis, promotion of angiogenesis, invasion of surrounding tissues and metastasis [1, 2].

Breast cancer is the most commonly diagnosed cancer in several sub-Saharan African countries, a shift from a trend in which cervical cancer was the leading cause of cancer-related deaths among women in Africa over the

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past decade [1]. The reasons for this shift still remain unclear but have a strong correlation with prevalent risk factors such as obesity, early menarche, late childbearing, and lifestyle associated with urbanization and economic development [3].

The generation of reactive oxygen species (ROS) during oxidative burst is considered as one of the major mechanisms by which phagocytes exert their tumoricidal functions [4]. Phagocytes such as macrophages undergo oxidative burst in response to antigenic stimuli with generation and release of different reactive oxygen metabolites thereby making oxidative burst as defense function [5]. Oxidative stress is closely linked with carcinogenesis due to the interplay of ROS in relation to other epigenetic factors in the induction, promotion, and modulation of breast and cervical cancer [6].

Nitric oxide (NO) is a short-lived pleiotropic regulator, that plays critical roles in numerous physiological as well as pathological processes [7]. Its role in tumor development is somewhat complex [8]. However, reported roles of NO such as genotoxic mechanisms, antiapoptotic effects, induction, and promotion of angiogenesis, limitation of host immune response against tumor, and promotion of metastasis has been implicated in various types of cancer [9] and NO tumor-promoting effect appears to be both time and concentration-dependent [10]. Overproduction of reactive oxygen (ROS) and nitrogen (RNS) species by phagocytes, namely neutrophils, may result in chronic inflammation and initiation of the multistage process of various cancer development including breast and cervical cancer [11].

Mitogen-activated protein (MAP) kinases in different cell types are involved in the production of extracellular polypeptides or glycoproteins called cytokines. Cytokine activity is influenced by the microenvironment in which they are produced and as such may have pro- (Th1) or anti-inflammatory (Th2) actions [12]. Tumor necrosis factor (TNF-α), an inflammatory cytokine that is highly expressed in breast and cervical carcinomas play an important role in the regulation of both induction and protection in breast and cervical cancer [13, 14], and apoptosis [15].

Abrus precatorius (family: Fabaceae) is a perennial, well-branched, twinning and climbing herb that bears a characteristic bright red colored seeds with a black blotch at the hilum [16]. It is endemic in the tropics and commonly known as Rosary bean. It is known as Otuobiribiri (Igbo-Ohafia), Idon zakara (Hausa), Oju ologbo (Yoruba) in Nigeria. The roots of *A. precatorius* contain proteins, glycosides, phenolic compounds, fatty acid, fatty acid esters, anthocyanins, and minerals [17]. *A. precatorius* has been reported from folklore to have potential antitumor properties [18]. Other reported ethnopharmacological and therapeutic activities include but not limited to antidiabetic [19, 20], anti-inflammatory [21] activities.

Based on the above mentioned considerations, this study evaluated the antiproliferative and immunosuppressive activities of methanolic crude extract and fractions of *A. precatorius* roots which will be able to simultaneously modulate human neutrophils' oxidative burst, restraining the inflammatory process, and inhibit the growth of breast (AU565) and cervical (HeLa) cancer cell lines. To underscore the broad-spectrum activity of the plant, two cancer cell lines of different origin were adopted for the study primarily due to their invasive metastatic nature.

Materials and methods

Plant material

Young roots of *A. precatorius* were collected from Imota Ikorodu, Nigeria. A voucher specimen (IFE-17655) was deposited at the Herbarium of Obafemi Awolowo University, Ile-Ife, Nigeria.

Extract preparation

Young fresh *A. precatorius* roots were thoroughly washed, oven-dried (Uniscope SM9053) at 40 °C for 96 h to a constant weight and pulverized. Methanol (70%) (1:10) crude extraction of pulverized *A. precatorius* roots was done for 48 h using a shaker water bath (Uniscope SM101) at 40 °C and filtered (Whatman No.1110 mm). The filtrate was concentrated using a Rotary evaporator (Stuart RE 300) to obtain the crude methanol extract, reconstituted in distilled water (1:5) and subjected to liquid-liquid partitioning using solvents of increasing polarity to obtain partially purified – n-hexane, ethyl acetate, n-butanol, and aqueous fractions respectively. The fractions were further concentrated to a constant weight under reduced pressure using a Rotary evaporator at 40 °C and freeze-drying for the aqueous fraction.

Phytochemical screening

To identify the chemical constituents and possible functional groups, *A. precatorius* fractions were subjected to Fourier Transform Infra-red (FTIR) spectroscopy measured on VECTOR22, Resolution 2 cm^{-1}(10 scans) and Gas chromatography-mass spectrometry (GCMS) analysis using Agilent 5975C gas chromatograph combined with inert XL EI/CI MSD and Triple-Axis Detector source at 270 °C at 70 eV. The injector was set at 270 °C with splitting ratio 1:30. A mass spectral survey was performed using the NIST mass spectral program. The concentrations of the identified compounds were calculated using area normalization over the FID response method.

Cytotoxicity screening

Cytotoxic activity of extract and fractions of *A. precatorius* roots, was evaluated in 96-well flat-bottomed microplates by using the standard MTT (3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyl-tetrazolium bromide) colorimetric assay. For this purpose, cell lines were cultured in Minimum Essential Medium Eagle, supplemented with 5% of fetal bovine serum (FBS), 100 IU/ml of penicillin and 100 μg/ml of streptomycin in $75cm^2$ flasks, and kept in 5% CO_2 incubator at 37 °C. 200 μL of cell suspension were seeded in round bottom 96 well plate at the density of 10,000 cell/well and incubated at 37 °C in 5% CO_2 incubator for 24 h. Exponentially growing cells were harvested, counted with a hemocytometer and diluted with the medium. Cell culture with the concentration of 6×10^4 cells/ml was prepared and introduced (100 μL/well) into 96-well plates. After overnight incubation, the medium was removed and 200 μL of fresh medium was added with different concentrations of 10–100 μg/mL of fractions and 0.23–30 μg/mL for 3 T3 cells.

After 48 h, 200 μL MTT (0.5 mg/ml) was added to each well and incubated further for 4 h. Subsequently, 100 μL of DMSO was added to each well. The extent of MTT reduction to formazan within cells was calculated by measuring the absorbance at 570 nm, using a microplate reader (Spectra Max Plus, Molecular Devices, CA, USA). The cytotoxicity was recorded as concentration causing 50% growth inhibition (IC_{50}) for cell lines [22]. Doxorubicin and Cyclohexamide served as standard drugs.

The percent inhibition was calculated by using the following formula:

$$\%Inhibition = 100 - \frac{(mean\ OD\ of\ test\ compound - mean\ OD\ of\ negative\ control)}{(mean\ OD\ of\ positive\ control - mean\ OD\ of\ negative\ control)} \times 100$$

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activities of extract and fractions of *A. precatorius* roots, was done using the method described by Sagar & Singh [23]. DPPH solution (95 μl, 300 μM) in Ethanol was mixed with test solution (5 μl, 500 μM). The reaction was allowed to progress for 30 min at 37 °C and absorbance monitored by the multiplate reader, SpectraMax340 at 517 nm. Upon reduction, the color of the solution faded (Violet to pale yellow). Percent Radical Scavenging Activity (%RSA) was determined by comparison with a DMSO containing control. The concentration that causes a decrease in the initial DPPH concentration by 50% is defined as IC_{50} value. The IC_{50} values of the fractions were calculated using EZ-Fit Enzyme kinetics software program (Perrella Scientific Inc. Amherst, MA, USA) and data reported as mean ± standard error of mean. N-acetylcysteine and Gallic acid were used as reference compounds.

Oxidative burst assay

The studies on human blood cells were performed after an approval from independent ethics committee, ICCBS, UoK, No: ICCBS/IEC-008-BC-2015/Protocol/1.0.

Luminol-enhanced chemiluminescence assay was performed, as described by Helfand et al. [24]. Briefly, 25 μL of diluted human whole blood / (1×10^6) isolated PMNs in $HBSS^{++}$ (Hanks Balanced Salt Solution, containing calcium chloride and magnesium chloride) [Sigma, St. Louis, USA] was incubated with 25 μL of various concentrations (0.1–250 μg/mL) of *A. precatorius* fractions. Control wells received $HBSS^{++}$ and cells, but no compounds. The test was performed in white half area 96 well plates [Costar, NY, USA], which was incubated at 37 °C for 15 min in the thermostat chamber of luminometer [Labsystems, Helsinki, Finland]. After incubation, 25 μL of serum-opsonized zymosan (SOZ) [Fluka, Buchs, Switzerland] and 25 μL of intracellular reactive oxygen species detecting probe, luminol [Research Organics, Cleveland, OH, USA] were added into each well, except blank wells (containing only $HBSS^{++}$). The level of the ROS was recorded in the luminometer in terms of relative light units (RLU) for 50 mins in the repeated scan mode. Ibuprofen was used as a standard drug.

Cell lines

The cell lines used in this study including AU565 (human breast adenocarcinoma, CRL-2351) and NIH-3 T3 (mouse embryonic fibroblast, CRL-1658) were purchased from ATCC, Manassas, USA, THP-1 (Human monocytic leukemia) and J774.2 (mouse macrophages) were purchased from ECACC, Salisbury, UK and HeLa cells (human cervical adenocarcinoma) were purchased from CLS, Germany by Dr. Panjwani Centre for Molecular Medicine and Drug Research (PCMD), International Center for Chemical and Biological Sciences) (ICCBS), University of Karachi, Karachi-75270, Pakistan. NIH-3 T3, HeLa and J774.2 cells were grown in DMEM supplemented with 10% Fetal bovine serum (FBS) and 1% penicillin/streptomycin and AU565 in ATCC modified Rosewell Park Memorial Institute (RPMI) medium supplemented with 90% FBS, 1% penicillin, 1% streptomycin. The cells were grown in 75 cc culture flask and upon reaching 75% confluency were harvested and used for experimental purpose. For cytotoxicity on NIH-3 T3 and HeLa (6×10^4 cells/mL), AU565 (6×10^4 cells/mL) and for NO assay from J774.2 (1×10^6 cells/mL) were used.

Nitric oxide assay

The mouse macrophage cell line J774.2 was cultured in 75 cc flasks IWAKI (Asahi Techno Glass, Japan) in DMEM Sigma-Aldrich (Steinheim, Germany) supplemented with 10% fetal bovine serum GIBCO (N.Y U. S) 1% streptomycin/penicillin. Flasks were kept at 37 °C in an atmosphere of humidified air containing 5% CO_2, cells were seeded in 96-well plate (10^6 cells/mL) and were induced by 30 μg/mL *Escherichia coli* lipopolysaccharide (LPS) (DIFCO Laboratories Michigan, USA). Three different concentrations of fractions (1, 10 and 100 μg/mL) were added simultaneously with LPS and the plate was incubated for 48 h at 37 °C in 5% CO_2. The FBS concentration was 5%. Nitrite accumulation in the culture supernatant was measured using the Griess reagent [25].

Cytokine production and quantification

Human monocytic leukemia cells THP-1 cells were maintained in RPMI-1640 containing 5.5 mmol/L glucose (BioM Laboratories, Chemical Division, Malaysia), 50 μmol/L mercaptoethanol (Merck Darmstadt, Germany), 10% FBS (fetal bovine serum), 2 mmol/L; L-glutamine (PAA Laboratories, GmbH, Pasching, Austria). Cells were grown in 75 cc flasks, harvested and 2.5×10^5 cells/mL was then plated in 24-well tissue culture plates. 20 ng/mL of phorbol myristate acetate (PMA), (SERVA, Heidelberg, Germany) was added followed by incubation for 24 h at 37 °C in 5% CO_2 to convert them into a macrophage-like cell. Cells were then stimulated with *E. coli* Lipopolysaccharide B, (DIFCO Laboratories, Michigan, USA) at a final concentration of 50 ng/mL and treated with fractions using three concentrations (1, 10 and 100 μg/mL) and then incubated for 4 h at 37 °C in 5% CO_2. The supernatants collected were analyzed for the level of TNF -α using the human TNF-α Duo Set ELISA (R&D Systems, Minneapolis, USA), and according to manufacturer's instructions [25].

Statistical analysis

Data from all experiments were statistically evaluated using one-way analysis of variance (ANOVA) on SoftMax Pro software and results expressed as mean ± standard deviation (SD) and mean ± standard error of mean (SEM). $P < 0.05$ at 95% and $p < 0.001$ at 99.9% confidence level was considered statistically significant for differences in mean and were obtained by Student's t-test analysis.

Results

Phytochemical analysis

Phytochemical screening revealed the presence of several chemical groups: flavonoids/isoflavones (5-Methoxy-3,7-dihydroxyflavanone, 4H-1-Benzopyran-4-one, 5,7-dihydroxy-2-(3-hydroxy-4,5-dimethoxyphenyl)-6,8-dimethoxy, 3-Hydroxy-2-(4-hydroxy-3-methoxyphenyl)-4H-chromen-4-one), triterpenes (lupeol, α-amyrin), aromatic carboxylic acids (Benzenepropanoic acid), aromatic alcohols (1H-Indole-3-(ethanol), as shown in Fig. 1 and Table 1.

Cytotoxicity screening

The antiproliferative activities of the crude extract and fractions of *A. precatorius* are presented in Table 2.

The results of the cytotoxicity of *A. precatorius* fractions against breast adenocarcinoma (AU565) cell line showed that ethyl acetate and aqueous fraction significantly ($p < 0.05$) inhibited breast adenocarcinoma proliferation (IC_{50} 18.10 ± 2.68 μg/mL, IC_{50} 46.46 ± 0.14 μg/mL respectively). Crude extract, hexane, and butanol fractions were found to be inactive against breast adenocarcinoma. The ethyl acetate and hexane fractions also showed significant (p < 0.05) inhibition of cervical cancer (HeLa) cell proliferation (IC_{50} 11.89 ± 0.63, 18.24 ± 0.16 μg/mL respectively). Crude extract, butanol, and aqueous fractions were found to be inactive against cervical cancer cells. All the fractions showed no cytotoxicity against 3 T3 murine fibroblast normal cells thus indicating their safety (Table 2).

In vitro antioxidant activity of *A. precatorius* fractions

The results of the DPPH Radical Scavenging Activity are presented in Table 3.

All assayed fractions of *A. precatorius* root methanol extract showed significant ($p < 0.001$) DPPH scavenging activity compared with standards Gallic acid and N-acetyl cysteine. Hexane fraction inhibited DPPH radical formation with significant (p < 0.001) IC_{50} 0.010 ± 0.002 mg/mL compared with the positive controls, making it the most active fraction in DPPH radical scavenging activity Methanolic crude extract inhibited DPPH formation with an IC_{50} of 0.087 ± 0.002 mg/mL; whereas the ethyl acetate, butanol, and aqueous fractions inhibited the formation of DPPH radicals with IC_{50} 0.079 ± 0.005 mg/mL, 0.098 ± 0.002 mg/mL, and IC_{50} 0.086 ± 0.002 mg/mL respectively compared with the standards Gallic acid (0.0032 ± 0.0001 mg/mL), and N-acetyl cysteine (0.0141 ± 0.0001 mg/mL) respectively (Table 3).

Immunomodulatory activities

The results of immunomodulatory activities are presented in Table 4.

Methanolic crude extract and ethyl acetate fraction of *A. precatorius* showed potent inhibition of whole blood ROS with IC_{50} of < 10 μg/mL respectively, whereas hexane (30.5 ± 0.3 μg/mL) and butanol (21.0 ± 0.5 μg/mL) fractions mildly inhibited whole blood ROS. Hexane,

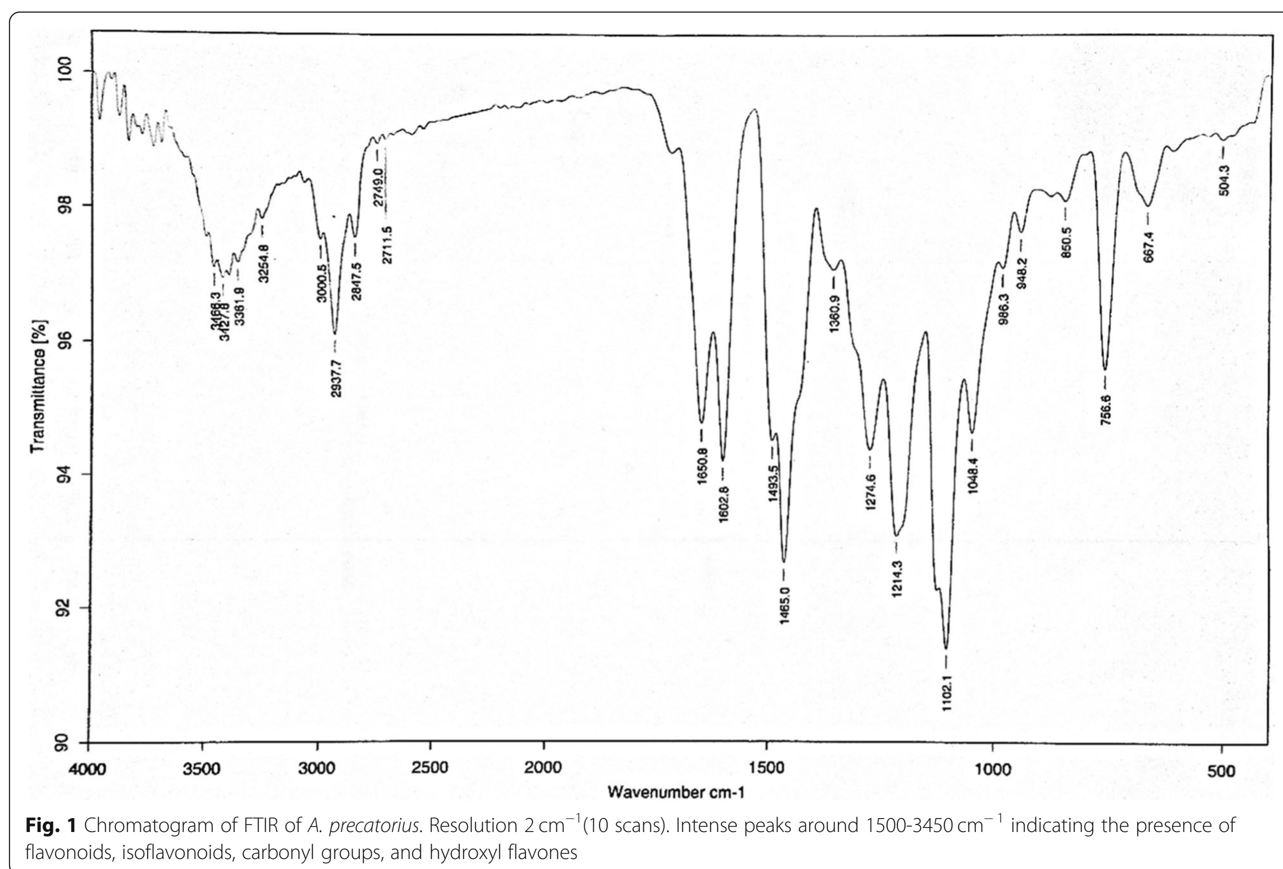

Fig. 1 Chromatogram of FTIR of *A. precatorius*. Resolution 2 cm^{-1}(10 scans). Intense peaks around 1500-3450 cm^{-1} indicating the presence of flavonoids, isoflavonoids, carbonyl groups, and hydroxyl flavones

ethyl acetate and butanol fractions of *A. precatorius* showed significant ($p < 0.001$) suppression of oxidative burst generated from zymosan activated polymorphonuclear cells (PMNs) (IC_{50} 0.6 ± 0.003 μg/mL, 0.6 ± 0.002 μg/mL, 6.1 ± 0.8 μg/mL respectively) and also found to significantly (p < 0.001) inhibit proinflammatory cytokine TNF-α with IC_{50} values of < 1 μg/mL for hexane and ethyl acetate and 5.74 ± 0.05 μg/mL for butanol fraction respectively. The hexane and ethyl acetate fraction also inhibited the NO production with IC_{50} values of < 1 μg/mL respectively. The aqueous fraction was found to be inactive (Table 4).

Discussion

Phytochemical screening showed that they are abundant in flavonoids, terpenes, alkaloids, and glycosides. Flavonoids, as well as terpenes, possess anticancer properties [26] through their effects on signal transduction in cell proliferation and angiogenesis [27]. Flavonoids have also been implicated in playing a major role in attenuating the development of tumors via their antioxidant effects [28]. Flavonoids may interfere with the activation of the proinflammatory nuclear factor-kB (NF-kB) and tumor activator protein-1 (AP-1) while inducing cell cycle arrest and apoptosis [29]. Flavonoids repress molecular targets that stimulate proliferation, inflammation, invasion, metastasis, and angiogenesis, and induce pro-apoptotic pathways [30, 31]. The observed cytotoxic activity may be attributed to the phytoconstituents such as flavonoids, and terpenes present in these fractions, which may have worked synergistically to exert these effects [32].

A handful of 'red line' events which propel tumor cells and their derivatized progenies into full-blown uncontrolled metastasis have made cancer a complex idiopathic disorder. Deregulation of cell proliferation alongside suppressed apoptosis, provide leverage for all cancer evolution and progression. Uncontrolled cell division is a primary determinant and underlying factor in the progression of cancer tumors. To evaluate fractions from root methanol extract of *A. precatorius* as a potential therapy for cancer, different fractions were assayed against human breast cancer cells (AU565) and cervical cancer (HeLa) cell lines. The antiproliferative effects were quantified in terms of cytotoxicity and IC_{50} values determined. One of the reliable criteria in the assessment of any anticancer drug is a decrease in tumor volume and viable tumor cell count and an increase in non-viable tumor cell count. The results of this study show an anticancer effect of *A. precatorius* fractions against breast adenocarcinoma and cervical cancer. The results reveal that the fractions were somewhat selective

Table 1 Identified compounds from methanolic crude extract of *A. precatorius* roots

Peak #	Compound	Formula	t_R (min)	Area %	Remarks
1	Benzoic acid	$C_7H_6O_2$	7.88	0.07	antimicrobial, antimutagenic, antiestrogenic, hypoglycemic, anti-inflammatory
2	1-Dodecanol	$C_{12}H_{26}O$	15.23	0.38	An insect attractant, pesticide
3	Benzaldehyde, 4-hydroxy-3,5-dimethoxy-	$C_9H_{10}O_4$	19.65	0.08	Antioxidant, antimicrobial and hypoglycemic
4	1-Hexadecanol	$C_{16}H_{34}O$	19.88	0.21	Emulsifier
5	Ethanol, 2-(dodecyloxy)-	$C_{14}H_{30}O_2$	20.76	0.13	
6	1H-Indole-3-ethanol	$C_{10}H_{11}NO$	21.83	0.46	Estradiol metabolism
7	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	26.83	5.31	Antioxidant, anti-inflammatory
8	Dibutyl phthalate	$C_{16}H_{22}O_4$	28.32	0.54	Antibacterial
9	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	28.58	0.75	Antibacterial, antifungal
10	Heptadecanoic acid, methyl ester	$C_{18}H_{36}O_2$	31.51	0.36	Antimicrobial
11	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	35.99	7.28	Antibacterial
12	11-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	36.44	5.08	Antimicrobial
14	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	37.89	1.27	Antimicrobial, anti-inflammatory, anticancer
15	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	38.22	0.46	Antibacterial
16	cis-Vaccenic acid	$C_{18}H_{34}O_2$	38.54	0.63	Antibacterial, hypolipidemic
17	Benzene, 1,1′-tetradecylidenebis-	$C_{26}H_{38}$	43.96	1.11	Antimicrobial
18	5-Methoxy-3,7-dihydroxyflavanone	$C_{16}H_{14}O_5$	47.19	2.66	Antioxidant, anti-inflammatory, anticancer
19	1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	47.29	1.71	Antimicrobial, antioxidant, anticancer
20	3-Hydroxy-2-(4-hydroxy-3-methoxyphenyl)-4H-chromen-4-one	$C_{16}H_{12}O_5$	49.87	7.48	Anticancer, antihypertensive, antioxidant
21	13-Docosenamide, (Z)-	$C_{22}H_{43}NO$	50.15	0.94	Antimicrobial
22	2,2,4-Trimethyl-3-(3,8,12,16-tetramethyl-heptadeca-3,7,11,15-tetraenyl)-cyclohexanol	$C_{30}H_{52}O$	51.09	0.51	
23	Benzaldehyde, 4-hydroxy-3,5-dimethoxy-, [(4-hydroxy-3,5-dimethoxyphenyl)methylene]hydrazone	$C_{18}H_{20}N_2O_6$	51.88	1.65	Indicator for laccase and peroxidase *activity*
24	4H-1-Benzopyran-4-one, 5,7-dihydroxy-2-(3-hydroxy-4,5-dimethoxyphenyl)-6,8-dimethoxy-	$C_{19}H_{18}O_9$	52.69	2.93	antibacterial
25	6,6,7-Trimethyl-9-oxo-3-oxabicyclo(3.3.1) nonane 2,4-dinitrophenylhydrazone	$C_{17}H_{22}N_4O_5$	53.59	9.16	
26	1,1′-Biphenyl-5-carboxylic acid, 2,3,4,4′-tetramethoxy-6′-methoxycarbon	$C_{19}H_{20}O_8$	53.76	1.13	Antibacterial
27	Silane, dimethyl(3-ethylphenoxy)tetradecyloxy-	$C_{24}H_{44}O_2Si$	54.71	41.09	Antimicrobial
28	Anthiaergosta-1,5,7,9-tetraene	$C_{28}H_{42}$	55.77	2.47	
29	Cyclohexan-1-one-3α,5β-diacetic acid, 2α-(5-hydroxy-2,4-dimethoxyphenyl)-, diethyl ester	$C_{22}H_{30}O_8$	56.14	0.83	Antibacterial
30	α-Amyrin	$C_{30}H_{50}O$	56.64	0.47	Antimicrobial, antifungal, anti-inflammatory, anticancer
31	Lupeol	$C_{30}H_{50}O$	57.31	0.89	Antiprotozoal, anti-inflammatory, antitumor
32	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, octadecyl ester	$C_{35}H_{62}O_3$	60.73	1.07	Antifungal. antimicrobial

in their activity against cancer cells with ethyl acetate fraction showing significant anticancer activity against HeLa cell lines and AU565 cell lines whereas hexane fraction showed significant inhibition against HeLa cells only. The crude extract did not show any observable inhibition in the assayed cancer cell lines and therefore indicate that by partial purification, the fractions contain some anticancer bioactive compounds which will be isolated and assayed in further study. Mild cytotoxicity of the crude extract was observed against 3 T3 murine fibroblast normal cells implying safety and selectivity. These findings are in agreement with a similar report in which dietary flavonoid luteolin inhibited the invasion of cervical cancer [29].

Table 2 Antiproliferative activities of methanolic crude extract and fractions of *A. precatorius* roots

	AU565	HeLa	3 T3
Fraction/Drug	IC_{50} ± SD	IC_{50} ± SD	IC_{50} ± SD
Crude	NA	NA	25.80 ± 0.91
Hexane	NA	18.24 ± 0.16	NA
Ethyl acetate	18.10 ± 2.68	11.89 ± 0.63	24.80 ± 2.19
Butanol	NA	NA	NA
Aqueous	46.46 ± 0.14	NA	NA
Doxorubicin	0.54 ± 0.04	0.80 ± 0.10	
Cyclohexamide			0.80 ± 0.10

NA: Inactive
Values are means of three replicates ± standard deviation (SD). Significant *p* value ($p < 0.05$) were obtained by Student's t test analysis. Composite treatments were compared using one-way analysis of variances (ANOVA)

One of the basic and routine assays which provide front line information on the antiradical activity of plant extracts is DPPH radical scavenging assay. The results of the DPPH scavenging activity from our work suggest that fractions of *A. precatorius* are good sources of antioxidants compounds. However, ethyl acetate and butanol fractions appear to be excellent sources of potent antioxidant secondary metabolites. This may be due to the abundance of various flavonoids - 4H-1-Benzopyran-4-one, 5,7-dihydroxy-2-(3-hydroxy-4,5-dimethoxyphenyl)-6,8-dimethoxy, 5-Methoxy-3,7-dihydroxyflavanone, 3-Hydroxy-2-(4-hydroxy-3-methoxyphenyl)-4H-chromen-4-one, triterpenes and other non-phenolic constituents with antioxidant effects such as α-Amyrin and Lupeol as shown by the phytochemical screening results. The observed IC_{50} values of the fractions which were a little higher compared with the standard drugs may be due to the crude nature of the fractions unlike the pure forms of the drugs. The findings of this study are congruent with a previous report by Mir et al. [33] in which leaf extracts of *A. precatorius* demonstrated antioxidant and antiproliferative activities.

Table 3 DPPH Radical Scavenging Activities of methanolic crude extract and fractions of *A. precatorius* roots

Fraction/Standard	IC_{50} ± SEM
Crude	0.087 ± 0.002
Hexane	0.010 ± 0.002
Ethyl Acetate	0.079 ± 0.005
Butanol	0.098 ± 0.002
Aqueous	0.086 ± 0.002
Gallic Acid	0.003 ± 0.0001
N-acetylcysteine	0.014 ± 0.0001

Values are expressed as mean ± SEM, $p < 0.001$

Table 4 Immunomodulatory activities of methanolic crude extract and fractions *A. precatorius* roots

Fraction/Drug	WB ROS	PMNs ROS	NO	TNF-α
	IC_{50} ± SD	IC_{50} ± SD	IC_{50} ± SD	IC_{50} ± SD
Crude	< 10.0	NT	NT	NT
Hexane	30.5 ± 0.3	0.6 ± 0.003	< 1	< 1
Ethyl acetate	< 10	0.6 ± 0.002	< 1	< 1
Butanol	21.0 ± 0.5	6.1 ± 0.8	NT	5.74 ± 0.05
Aqueous	12.0 ± 1.0	> 250	NT	NT
Standard (Ibuprofen)	11.2 ± 1.9			

NT: Not tested
Values are presented as mean ± SD of triplicates at $p < 0.001$ by Student's t test analysis. *WB* Whole Blood, *NO* Nitric Oxide, *TNF* Tumor necrosis factor, *ROS* Reactive Oxygen Species, *PMNs* Polymorphonuclear cells

Macrophages have been implicated in neoplasm destruction via infiltration into the tumor site and participation in inflammatory reaction. They generate reactive oxygen species through the oxidative burst process which has been fingered as a major mechanism for their antimicrobial and tumoricidal functions [34]. Agents such as zymosan used in this study can induce the sequence of oxidative reactions and are known as triggering agents; other substances which can modify the magnitude of the response are known as modulating agents. Therefore, the result of this study reveals the modulatory role of fractions of *A. precatorius* thus indicating their anti-inflammatory potentials.

Cytokines are critical for tumor immunosurveillance and have demonstrated therapeutic anti-tumor activity in murine models and in the clinical treatment of several human cancers where they play complex and often opposing roles in the development of the immune system, host defense, and tumor immunobiology [35, 36]. Tumor necrosis factor (TNF-α) is produced by macrophages and is involved in cell activation, co-stimulation, and inflammation processes. Hexane, ethyl acetate, and butanol fractions potently suppressed TNF-α with IC_{50} of < 1 μg/mL respectively for hexane and ethyl acetate and 5.74 μg/mL for butanol fraction. The result from this study showed that fractions of *A. precatorius* suppressed the expression of TNF-α, hence inhibiting signaling and communication among cancer cells and is congruent with a similar report by Kangsamaksin et al. [37].

Nitric oxide has been reported to have tumor-promoting roles via formation of toxic and mutagenic species, direct modification of DNA– strand breaks, oxidation and deamination of nucleic acids, inhibition of systems required to repair DNA lesions, and inhibition of cytochrome C release [9, 38]. The hexane and ethyl acetate fraction of *A. precatorius* inhibited NO production with IC_{50} values of < 1 μg/mL respectively, thus suggesting a reversal of antiapoptotic effect and genotoxic mechanisms of NO and immunomodulatory potentials of *A. precatorius*.

Conclusion

This work provides experimental evidence that methanol root extract of *A. precatorius* contains bioactive compounds that exhibit anticancer, antioxidant and immunomodulatory potential without toxic effects on normal cells. The modulatory effect on cytokines and antiproliferative activities might be due to the synergistic actions of bioactive compounds present in them especially the ethyl acetate and butanol fractions and therefore suggest chemotherapeutic use of *A. precatorius* in cancer treatment. However, extensive studies to characterize and elucidate the mechanism of action of the active principles present in these fractions on antiproliferative activities are currently in progress.

Acknowledgments
E.E. Okoro acknowledges the World Academy of Sciences for the advancement of Science in Developing Countries (TWAS), Trieste, Italy, for ICCBS-TWAS Fellowship at the H.E.J. Research Institute of Chemistry, ICCBS, University of Karachi, Karachi, Pakistan.

Authors' contributions
EEO, ORO and AJ conceptualized and designed the experiment. EEO, AJ and SS carried out the experiments. EEO wrote the manuscript. ORO, AJ, CMI and FDO read and approved the final manuscript for submission.

Funding
This research work was fully funded by the International Centre for Chemical and Biological Sciences, ICCBS, University of Karachi, Karachi, Pakistan and The World Academy of Science (TWAS) Trieste, Italy, for the advancement of Science in Developing Countries.

Availability of data and materials
Not applicable.

Ethics approval and consent to participate
The studies on human blood cells were performed after approval from independent ethics committee, ICCBS, UoK, No: ICCBS/IEC-008-BC-2015/Protocol/1.0.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 23 June 2019 Accepted: 11 December 2019
Published online: 20 December 2019

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