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Research Article
Antioxidant, Anti-inflammatory and Anticancer Activities of Ethanol Soluble Organics from Water Extracts of Selected Medicinal Herbs and Their Relation with Flavonoid and Phenolic Contents

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Abstract

Background and Objective: Medicinal herbs offer an important traditional way to prevent and cure several diseases such as cardiovascular disease, chronic inflammation and cancer as they contain bioactive compounds including those with antioxidant, immunomodulatory and anticancer activities. The purpose of this study was to determine biological activities of organics from hot water extracts of medicinal herbs and to obtain the correlation of activities with polyphenol contents. **Materials and Methods:** In this study, 16 herbs were selected based on their traditional medicinal uses and obtained their hot water extracts. Ethanol soluble organic molecules were separated from these extracts and their antioxidant, immunomodulatory and anticancer activities were assessed. Antioxidant activities were evaluated using $DPPH^{\bullet}$, $ABTS^{\bullet+}$ scavenging methods and ferric ion reducing assay. Total phenolic and total flavonoid contents of these extracts were estimated based on the Folin-Ciocalteu and aluminium chloride colorimetric methods. The immunomodulatory properties of the herbs were determined on the basis of their ability to inhibit NO and TNF-α production in LPS induced RAW 264.7 macrophages. Cell viabilities were determined using MTT assay. The anticancer activities were measured against five human cancer cell lines. All data was analysed using one-way ANOVA and Duncan's multiple range methods. **Results:** Organic molecules extracted from *Alpinae officinarum* (*A. officinarum*), *Artemisia annua*, *Cynanchum paniculatum*, *Lobelia chinensis* (*L. chinensis*), *Spatholobus suberectus* (*S. suberectus*), *Xanthium sibiricum* and *Amauroderma rugosum* (*A. rugosum*) have exhibited significant antioxidant activities and considerably inhibited the production of NO and TNF-α. Seven herbal extracts out of sixteen herbs studied showed highly significant anticancer activity against MCF7. The extract from *Rabdosia rubescens* displayed significant anticancer activities against three cancer cell lines. Observed biological activities of the extracts showed good correlation with their flavonoid contents. **Conclusion:** Extracts from *Akebia quinata*, *A. officinarum*, *Artemisia scoparia*, *L. chinensis*, *S. suberectus* and *A. rugosum* exhibited significant biological activities with large quantities of polyphenols. These herbs are potential candidates for the isolation of novel anticancer agents.

Key words: Traditional medicinal herbs, antioxidant activity, anti-inflammatory property, anticancer activity, polyphenols

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

It is well-known that medicinal herbs are extremely important sources of novel drugs and lead compounds for the discovery of new drugs[1-4]. Herbs continue to contribute to the development of important new classes of therapeutics including anticancer drugs and hence are beneficial to investigate their medicinal value using ever improving contemporary scientific tools[1]. Several medicinal herbs are available in the nature that have been used traditionally to treat different types of cancer[1,5]. However, large majority of these medicinal herbs are yet to be investigated comprehensively using modern scientific techniques.

Traditional Chinese medicinal (TCM) herbs have been used for the treatment of different types of cancers for thousands of years in Asian countries[2-9]. Many bioactive compounds isolated from medicinal herbs are in clinical use as anticancer agents[1]. An important class of therapeutic agents isolated from traditional herbs constitute flavonoids that display anticancer activities[1,10,11]. For example, scientific investigations demonstrate that flavone and flavopiridol isolated from *Dysoxylum binectariferum* Hook can prevent cancer formation by inhibition of several protein kinases such as cyclin-dependent kinases and tyrosine kinases[1]. Curcumin from *Curcuma longa* L. has been used in anticancer clinical trials due to its significant immunomodulatory properties[12] as well as protein kinase inhibition activities with minimal toxicity[13]. Genistein isolated from soybeans displays antiangiogenic effects by regulating the expression of vascular endothelial growth factor[10,14]. In addition, plant flavonoids such as quercetin, genistein, daidzein prevent cancer formation by their antioxidant and immunomodulatory activities[10,15]. Some of these compounds are in advanced phases of clinical trials for several types of cancers[10]. Abundant literature indicates the existence of several prenylated flavonoids which exhibit a broad spectrum of properties relevant for anticancer activity[16]. However, this important class of molecules have not fully been exploited to unravel their cancer-preventive properties and their therapeutic potential to treat cancer[16]. It is therefore, very important to undertake a detailed study on anticancer behaviour of polyphenols from traditionally used anticancer herbs.

As part of the research program initiated in our laboratory to discover anticancer agents from TCM herbs, sixteen traditionally known anticancer herbs (Table 1) have been carefully selected and investigated. Table 1 provides their traditional uses and biological activities. Available scientific studies and the TCM knowledge demonstrate that these sixteen medicinal herbs exhibit significant therapeutic

Table 1: Important anticancer herbs that are used by Chinese medicinal practitioners

Name of herbs	Chinese names	Family names	Traditional uses and scientific findings	References
Akebia quinata (Houtt) Decne.	Ba yue zha	Lardizabalaceae	Treatment of rheumatism, allergies diabetics and anticancer (Hep-G2)	Kang *et al.*[17]
Alpinae officinarum Hance	Gao liang jiang	Zingiberaceae	Anticancer and anti-allergic	Samarghandian *et al.*[18]
Artemisia annua L.	Qing gao	Asteraceae	treat malaria and cancer	Chu *et al.*[19]
Artemisia scoparia Waldst. and *Kit.*	Yin chan	Asteraceae	Antioxidant, treat malaria and cancer	Huang *et al.*[5]
Artemisia vulgaris L.	Ai ye	Asteraceae	Anticancer, inhibition growth of HL-60 leukemic cell line by mitochondria-dependent apoptosis	Saleh *et al.*[20]
Citrus reticulate Blanco	Ju ye	Rutaceae	Anticancer, inhibition growth of Human Gastric Cancer Cells SNU-668	Kim *et al.*[21]
Curcuma aromatica Salisb	Yu jin	Zingiberaceae	Anti-tumour, inhibition growth of lung carcinoma cells	Ma *et al.*[22]
Cynanchum paniculatum L.	Xu chang qing	Apocynaceae	Anticancer	Kim *et al.*[23]
Cyperus rotundus L.	Xiang fu	Cyperaceae	Antidiabetic, anti-obese, anti-platelet, anti-allergic, anti-inflammatory and anticancer	Peerzada *et al.*[24]
Lobelia chinensis Lour	Ban bian lian	Campanulaceae	Anti-mutagenic activity and Anti-microbial activity and anticancer,	Li *et al.*[25]
Polygonum cuspidatum Sieb. et Zucc	Hu zhang	Polygonacae	Anticancer, inhibition of growth of human skin melanoma cells	Lee *et al.*[26]
Rabdosia rubescens (Hamst.) Wuet.	Dong ling cao	Labiatae	Anticancer, apoptosis in human laryngeal cancer cells	Kang *et al.*[27]
Rheum palmatum L.	Da huang	Polygonaceae	Antitumor, anti-inflammatory, antimicrobial and hemostatic properties	You *et al.*[28]
Spatholobus suberectus Dunn.	Ji xie teng	Leguminosae	Anticancer	Wang *et al.*[29]
Xanthium sibiricum L.	Cang Er Zi	Asteraceae	Anti-cancer; anti-inflammatory responses via the inhibition of nuclear factor-κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3) in murine macrophages	Ju *et al.*[30]
Amauroderma rugosum	Jia zhi	Ganodermataceae	Anti-cancer	Chan *et al.*[31]

properties such as immunomodulatory, anticancer and other pharmacological activities[17-32]. It is a common practice in traditional Chinese medicine to use hot water extracts for cancer and other treatments[2]. Systematic scientific studies involving hot water extractable therapeutic agents from the selected sixteen TCM herbs is very limited[33-35]. Wealth of traditional knowledge of the selected herbs and the limited scientific understanding warrant further study on their hot water extracts.

This study therefore aims to identify most suitable medicinal plants from the selected sixteen herbs by a systematic investigation using modern scientific techniques. It was proposed to use a simple hot water extraction procedure in this research and test anti-inflammatory and anticancer efficacy of the extracts. Major significance of this research was to discover the best herbs that contain novel therapeutic agents which could ultimately substitute some of the existing chemotherapeutic agents that are expensive and also have severe side effects. Many cancer patients in developing countries cannot afford expensive chemotherapy treatment. Hence the discovery of novel therapeutics from the medicinal herbs is expected to provide tremendous benefit to the society.

The objectives of this study was to isolate ethanol soluble organic molecules from hot water extracts of the selected herbs and to determine their antioxidant, anti-inflammatory and anticancer properties. It was also aimed to correlate the bioactivities of these extracts with the total flavonoid and phenolic contents and to discover potential candidates for the isolation of chemotherapeutic agents. Results of this study has opened the way for bioactivity guided isolation of therapeutic agents from traditionally well-known anticancer herbs.

MATERIALS AND METHODS

This study was carried out during 2013-14 as part of the authors' quest for the discovery of novel anticancer agents. The research was mainly done at the School of Science and Health, Parramatta campus, Western Sydney University.

Collection of medicinal herbs associated with this study: The herbal plant materials were purchased from a Chinese Herbal Medical centre known as Bei Jing Tong Ren Tang located in Sydney (Australia). Sample specimen of all the herbs are stored in our research laboratory. This company has branches all over the world and is well known for their best practice in TCM. The herbs traded in Sydney Centre have approvals from both Australian and Chinese Governments. The company undertakes stringent authentication and quality control procedures for all the herbal materials supplied by them. Details of these selected herbs are presented in Table 1. All herbal samples were powdered and subjected to hot water extraction procedure.

Chemicals and reagents: The gallic acid, quercetin, sodium nitrate, aluminium chloride, DPPH$^{\bullet}$, ABTS$^{\bullet+}$, DMSO, F-C reagent, sodium carbonate, 95% ethanol, ascorbic acid, trypan blue 0.4%, tetra methyl benzidine, sulfanilamide, N-(1-1-napthyl) ethylenediamine dihydrochloride, Lipopolysaccharide (LPS) were purchased from Sigma (Australia) and Lomb Scientific Pty Ltd. (Australia). The foetal bovine serum (FBS), antibiotics and Dulbecco's modified eagle's medium (DMEM) with gluMax were purchased from BD bioscience. Tumor necrosis factor-α (TNF-α), ELISA standards and antibodies were purchased from BD bioscience (USA).

Isolation of ethanol solubles from hot water extracts: Thirty grams of dried medicinal herbs were ground to powder form and mixed well. The powdered plant material was subjected to hot water extraction using autoclave method (at 121°C for 2 h) and then cooled to laboratory temperature and the supernatant was separated by filtration. The supernatant was then treated with 95% ethanol (Extract: Ethanol = 1:4 volume ratio) for 24 h at 4.1°C. The ethanol supernatant was then collected by filtration using 0.45 μm Wittman filter paper. The solution was then freeze dried and kept in -20°C until further research[8]. The entire process of extraction is illustrated in Fig. 1.

Determination of total phenolic compounds: The Folin-Coicalteu (F-C) reagent was employed for the determination of total phenolic content[34-39]. The procedure followed for the assay was similar to the one published before[34,35,38]. A standard curve was built using different concentrations of gallic acid (0-1000 μg mL^{-1}) that was used as standard[34-39]. The regression of the standard curve gave a linear equation ($y = 0.004x+0.0496$, $R^2 = 0.9961$). The total phenolics in the ethanol soluble water extracts were calculated using the above equation. The samples were analysed in triplicates.

Determination of total flavonoids: The colorimetric method was employed for the determination of total flavonoids[38,39]. The procedure followed for the assay was based on the method published by Baba and Malik[38] and Zhishen *et al.*[39]. A standard curve was built using different concentrations of quercetin (0-1000 μg mL^{-1}) as standard[40]. The regression of the standard curve gave a linear equation ($y = 0.0006x-0.0033$, $R^2 = 0.9942$). The samples were analysed in triplicates.

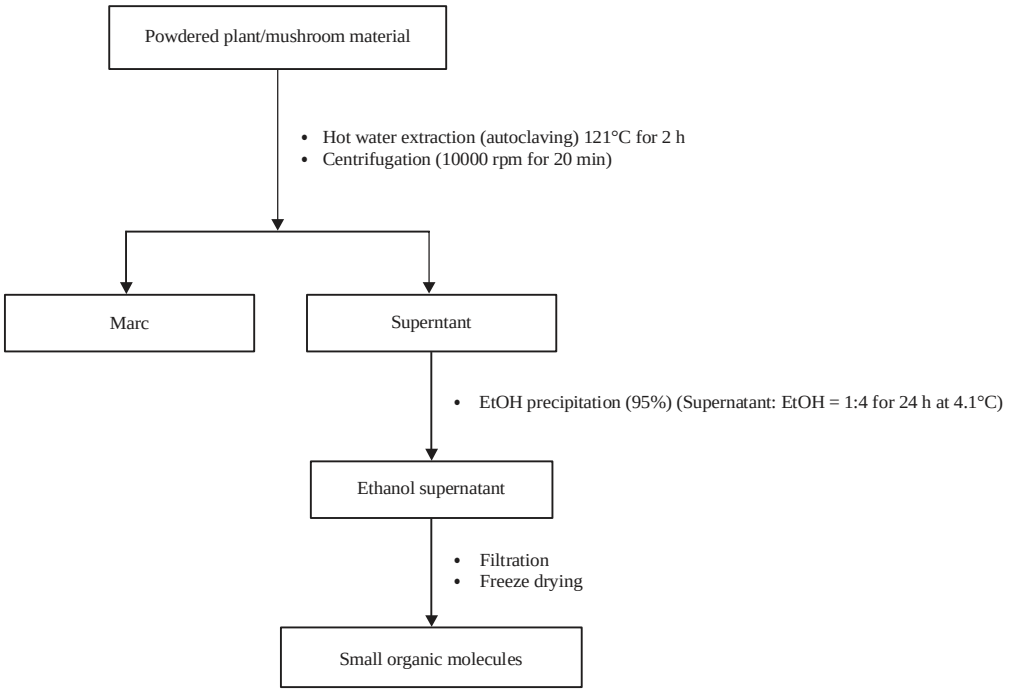

Fig. 1: Schematic diagram for the separation of ethanol soluble organics from hot water extracts of medicinal herbs

Bioactivity tests

DPPH·radical scavenging assay: Blois method[39,41-43] was employed in order to determine the DPPH· radical scavenging abilities of herbal extracts. The methodology employed for this assay was similar to the method published in the literature[34,35,39,41-43]. A standard curve was built using different concentrations of ascorbic acid solutions (in 60% methanol) in the range of 0-200 μM. The regression of the standard curve gave a linear equation ($y = -0.0016x+0.3515$ with $R^2 = 0.9648$). The free radical scavenging activities of herbal extracts were calculated as the ascorbic acid equivalent using the above equation.

$ABTS^{\cdot+}$ radical scavenging assay: A stock solution of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was prepared at a concentration of 7 mM using PBS buffer (pH 7.4). ABTS stock solution was mixed with potassium persulfate (2.45 mM) to initiate the formation of radical cations ($ABTS^{\cdot+}$). The reaction mixture was kept in a dark room for overnight to make sure that the radical formation is complete[41,42]. Absorbance of the $ABTS^{\cdot+}$ radical solution was adjusted to about 0.74 using PBS buffer (pH 7.4) to dilute the solution. About180 μL of $ABTS^{\cdot+}$ solution was added to 20 μL of herbal samples and incubated for about half an hour in a 96 well microtiter plate. The absorbance values of the incubated samples were then determined using UV spectrophotometer at 734 nm (Multiskan 141 EX, Thermo Electron, USA). Ascorbic acid was employed as positive control and PBS buffer (pH 7.4) was used as blank. A standard curve was built using different concentrations (0-400 μM) of ascorbic acid solutions in 60% methanol. The regression of the standard curve gave a linear equation ($y = -0.0023x+0.6996$ with $R^2 = 0.9852$). The free radical scavenging activities of the herbal extracts were calculated as the ascorbic acid equivalent using the above equation.

Ferric ions (Fe^{3+}) reducing antioxidant power: Herbal samples were prepared at different concentrations in the range of 0-1000 μg mL^{-1}. One hundred microliters of the sample was added with phosphate buffer (250 μL, 0.2 mol L^{-1}, pH 6.6) and then mixed with $K_3Fe(CN)_6$ (250 μL, 1% w/v). The solution was vortexed and incubated at about 50°C for 25 min. About 250 μL of 10% trichloroacetic acid (w/v) was then added to the incubated samples and the supernatant collected by centrifuging at 3500 rpm for about 10 min. The supernatant was then added with equivalent volumes of distilled water and $FeCl_3$ (0.1% w/v) and placed immediately into a spectrophotometer to measure the absorbance values at 700 nm. The samples were analysed in groups of three and when the analysis of one group has finished, the next group of three samples were mixed with $FeCl_3$ to avoid oxidation by air. Reducing power of ascorbic acid (standard) was also measured for comparison purposes[26,41,42].

Assays for immunomodulatory activities

Maintenance, preparation and activation of RAW 264.7 macrophages: Mouse macrophages (RAW 264.7 from Sigma-Aldrich) were first added to DMEM (culture medium containing 1% antibiotic and 5% FBS) and incubated for 4 days at 37°C in 5% CO_2. Cells were then diluted with the medium to achieve a density of 2×10^5 cells mL^{-1}. The approach followed to implement this assay was based on the procedure published in the literature[7,44,45,46].

NO production: The supernatant (100 µL) from each well was then carefully transferred into a new multiwell plate. Fifty microliters of sulfanilamide (1% w/v, dissolved in 5% H_3PO_4) was then added to supernatant and kept for 5 min at room temperature and 50 µL of Naphthyl ethylenediamine (0.1% w/v) was added to measure the concentration of NO as per the procedure outlined in previous publications[7,45,46]. Triplicate measurements were conducted.

Sodium nitrate was used as standard. The regression of the standard curve gave a linear equation (y = 0.0011x+0.3975 with R^2 = 0.9757) and the immunomodulatory activities of the herbal extracts were calculated using this equation.

TNF-α production: The supernatant (100 µL) from each well was carefully transferred into a new multiwell plate. The ELISA kit (BD Biosciences, San Jose, CA, USA) was then used to measure the concentration of TNF-α as per the procedure provided in the manufacturer's manual[34,35]. Regression of the standard curve gave a linear equation (y = 0.001x+0.1069 with R^2 = 0.9879). Immunomodulatory activities of the herbal extracts were calculated using the above equation. All measurements were conducted in triplicate.

Determination of cell viability by MTT assay: Viability of macrophage cells (RAW 264.7) were measured employing 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay[46]. Briefly, mouse macrophages were treated by herbal extracts and incubated at 37°C for 18 h. After that, supernatant was removed and then 100 µL of MTT solution (0.2 mg mL^{-1}, dissolved in DMEM medium) was added to each well and further incubated at 37°C for 4 h. Then, the supernatant was discarded and 50 µL of DMSO was added to each well to solubilise the crystalline formazan. The absorbance values were then measured at 595 nm. Cell viabilities were calculated using the following equation:

$$\text{Cell viability}(\%) = \frac{\text{OD of sample}}{\text{OD of pos control}} \times 100$$

where, positive control was mouse macrophages treated by DMEM Medium (without LPS).

Anticancer assays against various cancer cell lines: The cancer cell lines were cultured and incubated according to procedure outlined in a previous publication[6,47]. All the cancer cell lines studied in this research [MCF7 (Breast carcinoma), HT29 (Colon carcinoma), A549 (Lung carcinoma), Hep_G2 (Hepatocytes carcinoma) and MiaPAca2 (Pancreatic cancer)] were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Detailed methodology used for these assays was similar to that published before[6].

Optical density was determined at 570 nm using a spectrofluorometer. The percentage inhibition against various cancer cells was calculated using the following equation:

$$\text{Inhibition}\ (\%) = \frac{OD_{sample} - OD_{pos\ contr}}{OD_{Neg\ contr} - OD_{pos\ contr}} \times 100$$

where, $OD_{Neg\ Contr}$ is the optical density of the negative control and $OD_{Pos\ Contr}$ is the optical density of the positive control. The culture medium containing DMSO (1%) is used as negative control and the medium with 2 mM MMS was used as positive control.

Statistical analysis: All data was measured and calculated in triplicate and Mean±SD. The group mean was compared using a one-way analysis of variance (ANOVA) and Duncan's multiple range tests. Statistical calculations were done using OriginPro 8.5 (OriginLab Corporation, Northampton, USA) and Excel 2016 (Official Microsoft, USA). The data were considered to be statistically significant if $p<0.05$.

RESULTS AND DISCUSSION

Chemical composition: Total phenolic and flavonoid contents of ethanol soluble organics from hot water extracts of selected 16 herbs were measured using Folin-Coicalteu and aluminium chloride assays, respectively and the results are presented in Table 2. The phenolic and flavonoid contents were expressed in gallic acid equivalent (GAE mg g^{-1}) and quercetin equivalent (QE mg g^{-1}) of the extract per gram of the dried plant material. The highest phenolic contents were observed in the extracts of *P. cuspidatum* (14.33±0.14 mg g^{-1}). The flavonoid contents were relatively larger in all the extracts compared to their phenolic contents (Table 2). Highly significant flavonoid contents were found in *P. cuspidatum* (24.86±4.19 mg g^{-1}), *X. sibiricum* (20.61±1.67 mg g^{-1}), *A. quinata* (20.46±1.67 mg g^{-1}), *A. vulgaris* (19.09±0.96 mg g^{-1}) and *A. rugosum* (17.85±1.67 mg g^{-1}). The herbs *A. officinarum*, *A. annua*, *A. scoparia* and *S. suberectus* also had significant levels of flavonoid contents.

Antioxidant activities

DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activities: In this study, the radical scavenging activities of the herbal extracts were evaluated using DPPH$^{\bullet}$ and ABTS$^{\bullet+}$radicals. The results of free radical scavenging capacity of the extracts are presented in Table 2. Most of the plant extracts showed significant scavenging activity. As can be seen from the Table 2, highly significant DPPH$^{\bullet}$ scavenging activities were shown by the extracts of *R. rubescens*, *P. cuspidatum*, *A. officinarum*, *A. quinata*, *A. scoparia*, *A. vulgaris*, *A. annua*, *S. suberectus, X. sibiricum* and *A. rugosum* for which the activities were greater than 180 μM ascorbic acid equivalent. High ABTS$^{\bullet+}$ scavenging activities were displayed by the extracts of *R. rubescens*, *A. quinata*, *A. officinarum*, *A. scoparia*, *A. annua*, *A. vulgaris*, *P. cuspidatum* and *A. rugosum* which were greater than 270 μM ascorbic acid equivalent.

Fe^{3+} reducing power: The Fe^{3+} reducing power of the extracts were also measured as part of evaluating the antioxidant potentials of the herbal extracts. The results of concentration dependant reducing power of the extracts are presented in Table 3. The extracts from *A. officinarum*, *A. vulgaris*, *R. palmatum* and *S. suberectus* showed significant reducing ability.

Table 2: Antioxidant activities of the extracts from sixteen Chinese medicinal herbs along with their total phenolic and flavonoid contents

Name of herbs	Phenolic content (GAE mg g^{-1})*	Flavonoid content (QE mg g^{-1})*	DPPH scavenging activity (Ascorbate equivalent (μM)#	ABTS scavenging activity (Ascorbate equivalent (μM)#
Akebia quinata (*Houtt.*) *Decne.*	8.87±0.14	20.46±1.67	188.65±0.36	302.43±0
Alpinae officinarum Hance	5.20±0.14	14.38±1.67	190.11±0.36	283.59±0.25
Artemisia annua L.	7.19±0.29	12.98±4.41	185.31±0.63	296.35±0
Artemisia scoparia Waldst. and *Kit.*	3.77±0.25	11.51±0.96	182.69±0.63	263.74±0
Artemisia vulgaris L.	6.09±0.52	19.09±0.96	185.06±0.36	294.17±0
Citrus reticulata	4.68±0.75	4.39±1.67	145.31±0.63	203.16±3.7
Curcuma aromatica Salisb	1.82±0.29	2.84±0.47	125.31±0.72	142.43±0.25
Cynanchum paniculatum L.	0.12±0.63	1.36±0.96	112.40±2.89	133.16±0.25
Cyperus rotundus L.	3.41±0.29	4.66±0	154.06±0.63	192.43±0
Lobelia chinensis Lour	2.45±0.14	8.13±1.92	174.31±1.08	208.43±0
Polygonum cuspidatum Sieb. et Zucc	14.33±0.29	24.86±4.19	189.69±0.72	298.81±0.25
Rabdosia rubescens (*Hamst.*) *Wuet.*	8.40±0.66	9.51±3.47	198.90±2.25	303.74±0
Rheum palmatum L.	0.16±0.29	2.16±1.92	118.44±0.63	121.86±0
Spatholobus suberectus Dunn.	5.62±1.28	15.35±0.96	187.19±1.65	228.67±0.25
Xanthium sibiricum L	6.22±0.14	20.61±1.67	184.69±1.25	238.81±0.5
Amauroderma rugosum	8.18±0.38	17.85±1.67	176.56±2.86	275.19±5.02

*Total phenolic and flavonoid content were expressed in gallic acid and quercetin equiv mg^{-1}, respectively; #DPPH, ABTS free radical scavenging activity was expressed as equivalent of ascorbic acid (μM); All values are mean of triplicate determination±standard deviation

Table 3: Ferric ions reducing power of the extracts isolated from sixteen medicinal herbs

	Absorbance at 700 nm				
Herbs/standard	1000 (μg mL^{-1})	500 (μg mL^{-1})	250 (μg mL^{-1})	125 (μg mL^{-1})	62.5 (μg mL^{-1})
Vit C	1.707±0.006	0.706±0.001	0.331±0.001	0.193±0	0.127±0.001
Akebia quinata (*Houtt.*) *Decne.*	0.285±0.001	0.136±0.002	0.074±0.002	0.035±0.003	0.014±0.004
Alpinae officinarum Hance	1.138±0.001	0.741±0.005	0.345±0.001	0.214±0.002	0.106±0.002
Artemisia annua L.	0.37±0.001	0.257±0.001	0.133±0.002	0.066±0.002	0.033±0.002
Artemisia scoparia Waldst. and *Kit.*	0.349±0.001	0.234±0.024	0.121±0.004	0.05±0.001	0.027±0.001
Artemisia vulgaris L .	1.157±0.003	0.734±0.001	0.316±0.002	0.184±0.001	0.086±0.003
Citrus reticulata Blanco	0.269±0.001	0.153±0.003	0.075±0.001	0.052±0.001	0.048±0
Curcuma aromatica Salisb	NA	NA	NA	NA	NA
Cynanchum paniculatum L.	0.444±0.003	0.254±0.002	0.153±0.004	0.077±0.002	0.035±0.001
Cyperus rotundus L	0.274±0.002	0.137±0.002	0.042±0.002	0.024±0	NA
Lobelia chinensis Lour	NA	NA	NA	NA	NA
Polygonum cuspidatum Sieb. et Zucc	0.876±0.002	0.518±0.002	0.281±0.007	0.097±0.001	0.017±0.003
Rabdosia rubescens (*Hamst.*) *Wuet.*	0.726±0.002	0.412±0.003	0.208±0.001	0.108±0.001	0.058±0.001
Rheum palmatum L.	1.091±0.001	0.709±0.002	0.407±0.001	0.216±0	0.122±0.001
Spatholobus suberectus Dunn.	1.962±0.045	0.976±0.003	0.447±0.001	0.285±0.001	0.192±0.005
Xanthium sibiricum L.	0.504±0.003	0.247±0.002	0.127±0.003	0.076±0.002	0.017±0.001
Amauroderma rugosum (Blume and T. Nees) Torrend	0.296±0.002	0.204±0.001	0.088±0.002	0.038±0.002	0.014±0.002

NA: No activity, $p<0.05$, Mean±SD

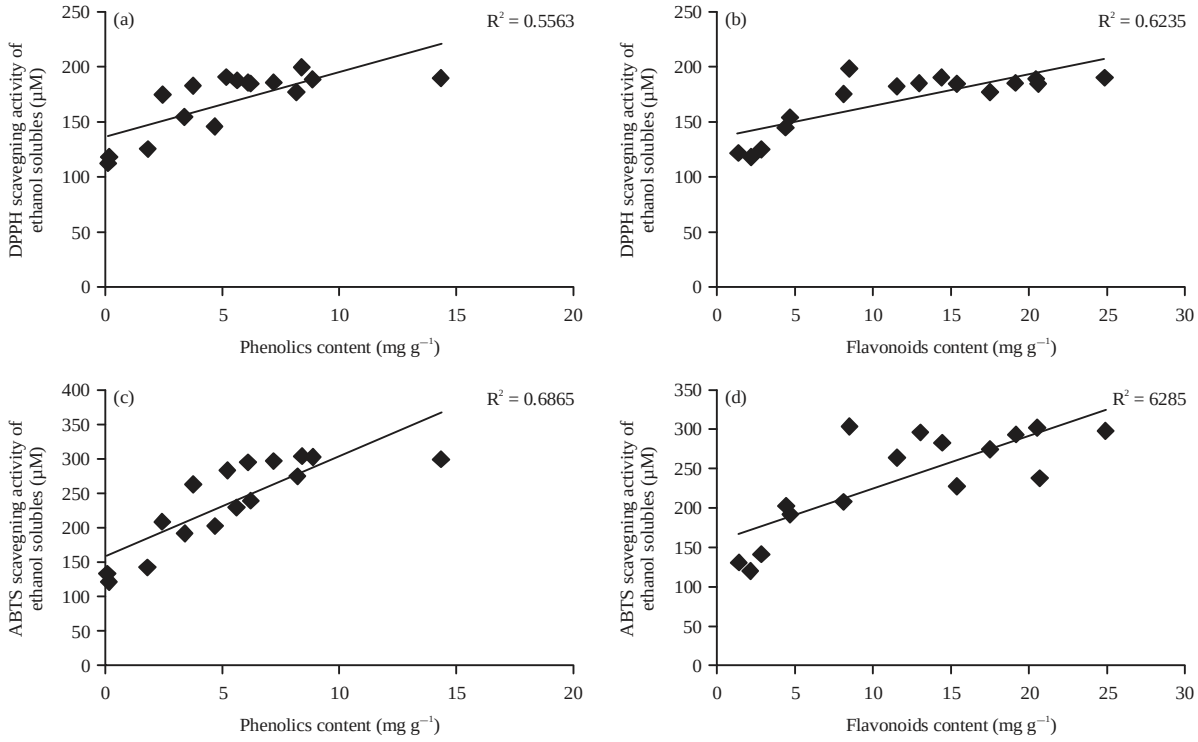

Fig. 2(a-d): Correlation between antioxidant activity and the total phenolic and flavonoid contents in the extracts, (a) $DPPH^{\bullet}$ vs. phenolics, (b) $DPPH^{\bullet}$ vs. flavonoids, (c) $ABTS^{\bullet+}$ vs. phenolics and (d) $ABTS^{\bullet+}$ vs. flavonoids

Antioxidants eliminate oxidative stress by scavenging free radicals that cause damage to DNA and lead to inflammation and cancer formation[48,49]. Natural antioxidants from TCM herbs are attractive alternatives to synthetic antioxidants[48,49]. Extracts from several selected herbs studied in this research have exhibited significant radical scavenging as well as Fe^{3+} reducing abilities. The most potent extracts include *A. officinarum*, *A. vulgaris*, *P. cuspidatum*, *R. rubescens*, *S. suberectus* and *X. sibiricum*. It is therefore, expected that these herbs are potential candidates for the isolation of antioxidant compounds. A correlation of antioxidant activities of the selected herbal extracts and their polyphenol contents are presented below.

Correlation plots were developed in order to reveal the relationship between the antioxidant activities and polyphenol contents of the extracts (total phenolics and flavonoids) (Fig. 2). $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activities of the extracts showed significant correlation (R^2 is greater than 0.55) with total phenolic contents (Fig. 2a, c) and also with the total flavonoid contents (Fig. 2b, d). The results presented above indicate that the total phenolic and flavonoid contents are important contributors to the antioxidant activities of the ethanol soluble water extracts from herbal medicine and this observation is in agreement with the literature[8,34,35]. The observed correlations of radical scavenging activities of the extract from *A. rugosum* with total phenolics are in agreement with those reported in the literature[49]. Recent studies also indicate that polyphenols isolated from *A. officinarum*, *A. quinata*, *A. annua* are potential candidates with significant antioxidant activities[40,50,51].

Anti-inflammatory activities: Literature demonstrates that increased production of NO and TNF-α can cause inflammation[52,53]. This study investigated the abilities of the herbal extracts to inhibit the production of NO and TNF-α in LPS-induced RAW 264.7 macrophages. The inhibition activity was expressed in terms of IC_{50} values and the results are presented in Table 4. It can be seen that many herbal extracts showed inhibitory activity against the production of Nitric oxide (NO). The extracts from *A. annua*, *A. officinarum*, *A. vulgaris*, *A. rugosum*, *L. chinensis*, *S. suberectus* and *X. sibiricum* significantly down regulated the NO production with IC_{50} values that are less than 229 µg mL^{-1}. Results presented in Table 4 also indicate that the extracts from *A. annua*, *A. officinarum*, *A. vulgaris*, *C. rotundus* and *L. chinensis* display significant inhibitory activity against TNF-α production with IC_{50} values less than 327 µg mL^{-1}.

Table 4: Anti-inflammatory activities of the extracts from selected medicinal herbs

Name of herbs	IC_{50} for the inhibition of NO production (μg mL^{-1})*	Cell viability (% of cell survival)&	IC_{50} for the inhibition of TNF-α production (μg mL^{-1})	Cell viability (% of cell survival)&
Akebia quinata.	687.12±5.22	94.4±1.47	484.64±2.42	89.17±3.55
Alpinae officinarum	229.31±4.61	96.8±1.73	348.67±2.43	91.47±10.09
Artemisia annua	47.42±0.78	106.43±4.04	156.67±1.78	87.57±8.99
Artemisia scoparia.	245.13±3.99	94.5±3.5	388.49±1.92	86.07±2.29
Artemisia vulgaris	108.15±6.21	97.77±1.17	128.42±0.48	95.43±0.93
Citrus reticulata	333.92±1.92	92.73±3.31	343.08±4.90	74.53±3.52
Curcuma aromatica	NA		NA	
Cynanchum paniculatum	NA		NA	
Cyperus rotundus	217.72±2.54	65.73±2.97	271.58±4.96	78.20±1.54
Lobelia chinensis	159.95±2	87.33±2.08	143.76±5.34	77.03±1.96
Polygonum cuspidatum	265.14±4.95	76.53±5.71	330.37±5	88.43±0.45
Rabdosia rubescens.	253.42±2.88	96.5±5.07	430.83±3.44	93.50±7.13
Rheum palmatum	NA		NA	
Spatholobus suberectus	200.94±3.35	88.63±0.32	301.69±1.17	57.00±2.58
Xanthium sibiricum	148.13±3.01	79.73±10.71	313.91±5.02	68.50±0.50
Amauroderma rugosum	81.31±4.47	88.77±2.87	327.38±3.67	94.87±1.79

*Inhibition of NO and TNF-α production was expressed in terms of IC_{50} values, $p<0.05$; &Cell viabilities were measured at a concentration of 1 mg mL^{-1} of herbal extracts, NA: No activity, Mean±SD

Concentration dependant anti-inflammatory activities of most active herbs are shown in Fig. 3a and b. Extracts from *A. annua* and *A. vulgaris*, have displayed high activity against TNF-α production. The herbs *C. rotundus* and *L. chinensis* have also displayed highly significant activities against TNF-α production (Fig. 3b). The extracts from *A. annua*, *A. vulgaris*, *A. rugosum* and *X. sibiricum* have shown highly significant concentration dependant inhibition of NO production (Fig. 3a).

Cell viabilities: Effects of the extracts from sixteen medicinal herbs on the viability of mouse macrophages are given in Table 4. Cell viabilities were measured at 1 mg mL^{-1} of the extracts. It is clear from these results that, all of the extracts showed significant cell viabilities (57% or better). These results indicate that the extracts of chosen herbs exhibit low toxicity and this is consistent with literature reports that the water extracts of medicinal herbs display least toxicity[34,35].

It should be noted at this point that, over production of NO results in damage to lipid cell membrane that may lead to cancer formation[48,49,53,54]. In such situations, the agents that inhibit the production of NO are beneficial. Many of the herbal extracts studied in this research exhibited excellent inhibitory activity against the production of NO. These results suggest that the herbal extracts contain anti-inflammatory compounds. Literature demonstrates that polyphenols are important class of anti-inflammatory molecules[11,55]. Correlation of the observed anti-inflammatory activities of the extracts with their polyphenol contents is presented below.

It is interesting to note from the results that there is a good correlation between the anti-inflammatory activities and total phenolic and flavonoid contents. For instance, *A. officinarum*, *A. annua*, *X. sibiricum*, *S. suberectus* and *A. rugosum* have significantly inhibited the production of NO/TNF-α (low IC_{50} values) and also have significant levels of total phenolic and flavonoid contents (Table 2, 4). Other herbs such as *C. aromatic*, *C. paniculatum* and *R. palmatum* were found to contain low levels of phenolic and flavonoid contents and did not display anti-inflammatory activity (Table 2, 4). These results are in agreement with the previous studies that phenolics and flavonoids display anti-inflammatory activities[55]. Literature demonstrates that polyphenols isolated from medicinal herbs display strong anti-inflammatory activities. For instance, polyphenolic compounds, namely, galangin and 5-hydroxy-7-(4"-hydroxy-3"-methoxyphenyl)-1-phenyl-3-heptanone, isolated from *A. officinarum* significantly inhibited the production of pro-inflammatory factor (COX-2)[40]. On the other hand, the anti-inflammatory activities of two of the herbs investigated in this study are not consistent with the total phenolic and flavonoid contents. For instance, *C. reticulata* and *C. rotundus* are found to have significant anti-inflammatory activities but these plants contain low levels of phenolics and flavonoids. Hence, it is concluded that chemical constituents other than phenolics and flavonoids may also be responsible for the anti-inflammatory properties of such plants[53,54].

Anticancer activities: The anticancer activities of the extracts from sixteen Chinese medicinal herbs were evaluated against five human cancer cell lines which included A549 (lung carcinoma), MCF7 (breast carcinoma), HT29 (colon carcinoma), Hep_G2 (Hepatocites carcinoma) and MiaPAca2 (Pancreatic Cancer). These results are expressed in terms of IC_{50} values and presented in Table 5.

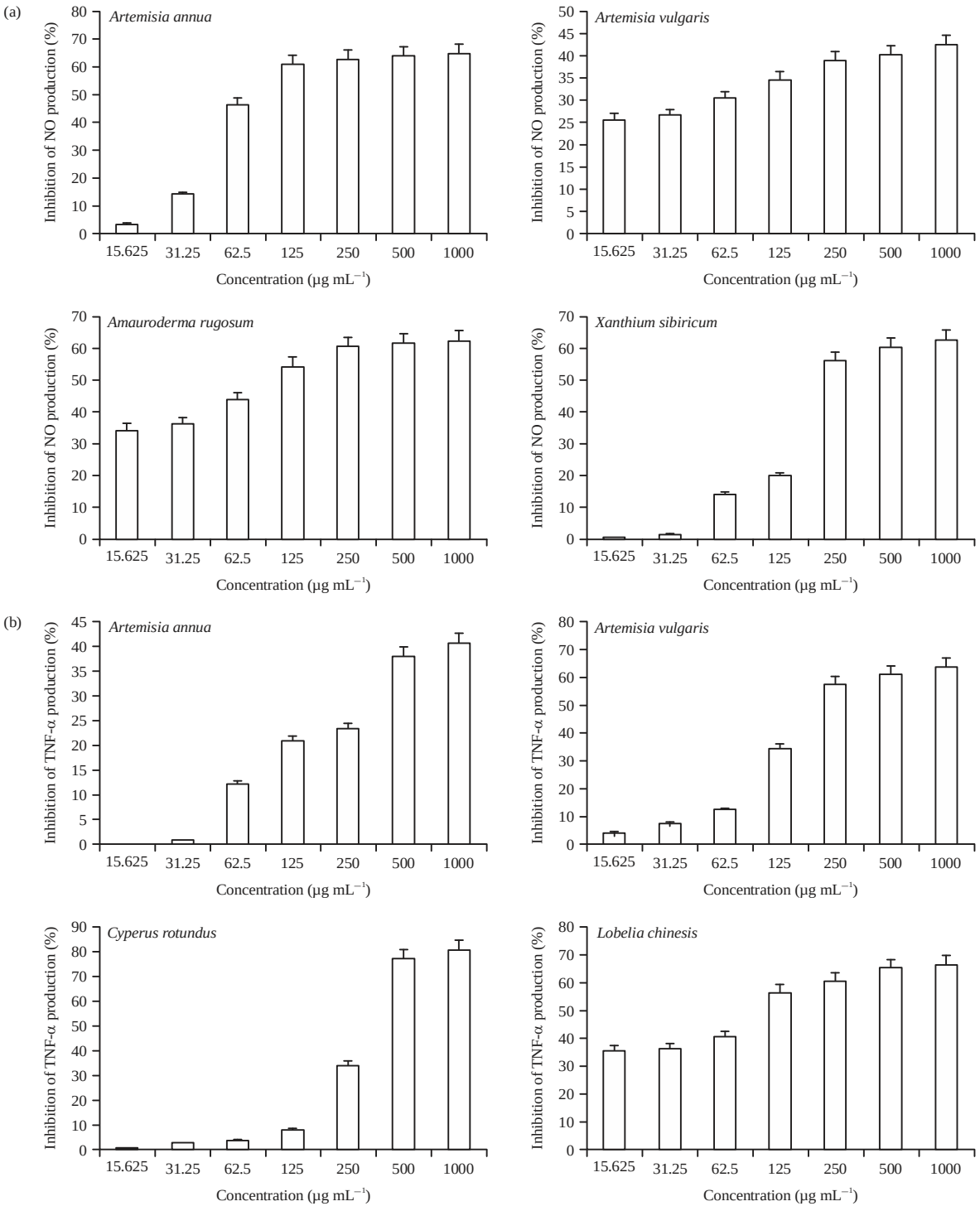

Fig. 3(a-b): Concentration dependant immunomodulatory activities of most active extracts from the selected herbs, (a) NO production and (b) TNF-α production

Values are expressed as Mean±SD (n = 3): $p<0.05$

It is interesting to note from the results (Table 5, Fig. 4a) that several extracts, namely, *A. officinarum*, *A. scoparia*, *C. aromatic*, *L. chinensis*, *R. rubescens*, *S. suberectus* and *A. rugosum* have significantly inhibited MCF7 (breast carcinoma) cell growth. The extract from *R. rubescens* displayed significant anticancer activities against three cancer

Table 5: *In vitro* cytotoxicity (IC_{50}) of the extracts from herbs against five cancer cell lines

	IC_{50} (μg mL^{-1})*				
Name of Herbs	A549	MCF7	HT29	MiaPAca2#	Hep_G2
Akebia quinata.		14.65±0.94			
Alpinae officinarum		14.64±1.34			
Artemisia annua					
Artemisia scoparia		6.86±2.6			
Artemisia vulgaris					
Citrus reticulata					
Curcuma aromatica		36.23±2.53			
Cynanchum paniculatum					
Cyperus rotundus					
Lobelia chinensis		11.75±3.91			
Polygonum cuspidatum					
Rabdosia rubescens.	15.33±0.22		22±8.64		10.75±2.91
Rheum palmatum					
Spatholobus suberectus		10.65±1.3			
Xanthium sibiricum					
Amauroderma rugosum		11.75±5.64			

#None of the extracts were active against Hep_G2 cell lines, *Smaller IC_{50} value indicates high activity, Mean±SD

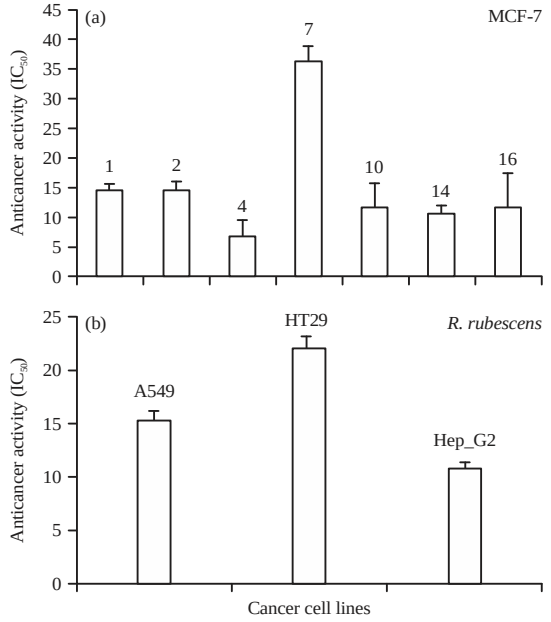

Fig. 4(a-b): Anticancer activities (IC_{50}) of the extracts (a) Extracts from *Akebia quinata* (1), *Alpinae officinarum* (2), *Artemisia scoparia* (4), *Curcuma aromatica* (7), *Lobelia chinensis* (10), *Spatholobus suberectus* (14) and *Amauroderma rugosum* (16) against breast cancer cell line (MCF-7) and (b) Extracts from *Rabdosia rubescens* against three different cancer cell lines (A529, HT29 and Hep_G2)

cell lines, namely, A549 (lung carcinoma), HT29 (colon carcinoma) and Hep_G2 (Hepatocites carcinoma) (Table 5, Fig. 4b, 5a). Figure 5a-c presents the results of concentration dependant anticancer activities of the three most active extracts (*A. scoparia*, *R. rubescens* and *S. suberectus*).

A review of literature[48,49] offers strong evidence that prolonged oxidative stress leads to inflammation and tissue damage that can potentially cause cancer formation and growth. Therefore, the agents that simultaneously possess antioxidant, anti-inflammatory and anticancer properties are of great importance for the prevention and treatment of cancer[10-13]. Some of the herbal extracts investigated in this study exhibited these important biological activities and hence are extremely suitable candidates for the isolation of anticancer agents. Polyphenols in general and flavonoids in particular are known in the literature to be highly potential anticancer agents[10-13,52]. Correlation of observed anticancer activities of herbal extracts with their polyphenol contents is discussed below.

Many of the herbal extracts investigated in this study showed significant correlation of anticancer activities with their phenolic and flavonoid contents. For example, *A. quinata*, *A. officinarum*, *A. scoparia*, *L. chinensis*, *R. rubescens*, *S. suberectus* and *A. rugosum* contained medium to high quantities of polyphenols and exhibited significant anticancer activities. This observation is consistent with the literature that flavonoids possess significant anticancer activities[10-13]. It should be noted that some of the herbal extracts investigated in this study contain polyphenols (Table 2) but did not display any anticancer activity (e.g. *A. annua*, *A. vulgaris*, *C. reticulate*, *C. rotundus*, *P. cuspidatum* and *X. sibiricum*). This may be due to the fact that the flavonoids present in these extracts may not be structurally relevant to anticancer activities[10,56,57].

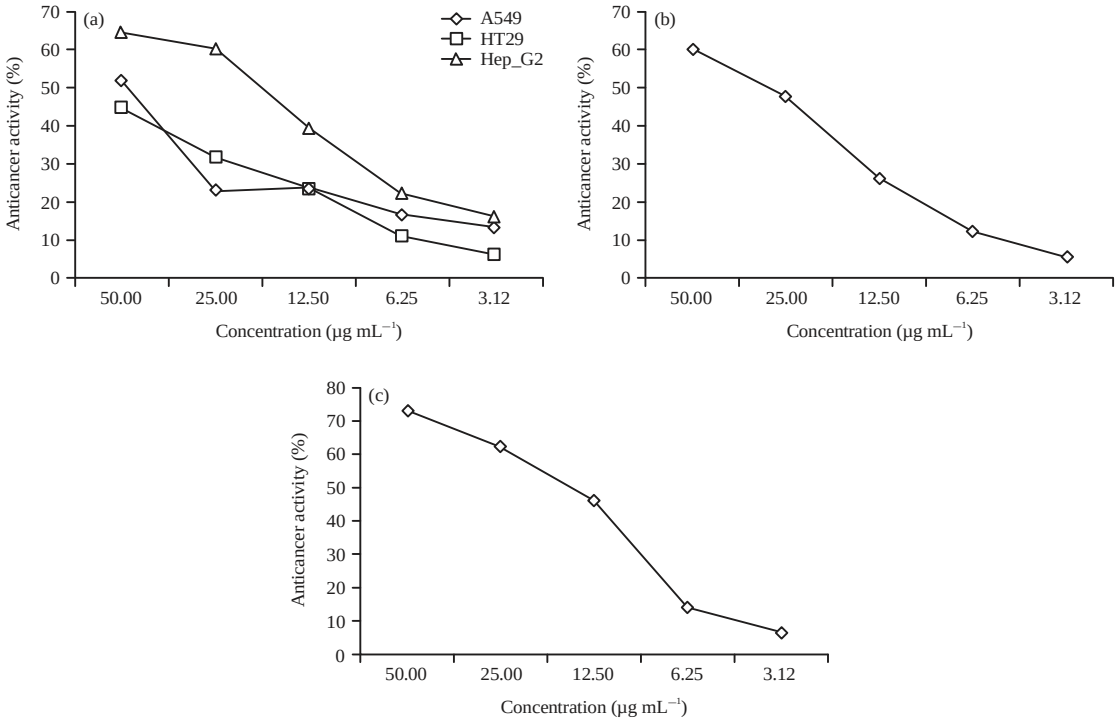

Fig. 5(a-c): Dose dependant variation of anticancer activities of the extracts from the three most active herbs, (a) Extracts from *Rabdosia rubescens* against three different cancer cell lines (A529, HT29 and Hep_G2), (b) Extracts from *Artemisia scoparia,* against breast cancer cell line (MCF-7) and (c) Extracts from *Spatholobus suberectus* against breast cancer cell line (MCF-7)

Table 6: Important anticancer herbs identified in this research together with their polyphenol contents

Name of the herbs	Total quantity of polyphenol	Antioxidant activities	Anti-inflammatory activities	Anticancer activities	Comment
Akebia quinata	+++	+++	+	+++	Highly potential candidate*
Alpinae officinarum	++	+++	++	+++	Highly potential candidate
Artemisia annua	++	+++	+++		
Artemisia scoparia	++	++	++	+++	Potential candidate
Artemisia vulgaris	+++	+++	+++		
Citrus reticulata		+	+		
Curcuma aromatica				+	
Cynanchum paniculatum					
Cyperus rotundus		++	+++		
Lobelia chinensis	+	++	+++	+++	Potential candidate
Polygonum cuspidatum	+++	+++	++		
Rabdosia rubescens	+	+++	++		
Rheum palmatum					
Spatholobus suberectus	++	++	+++	+++	Highly potential candidate
Xanthium sibiricum	+++	++	+++		
Amauroderma rugosum	+++	+++	+++	+++	Highly potential candidate

+++: Extremely high activity or large quantity of polyphenols; ++: Significant activity or significant quantity of polyphenols; +: Average activity or average quantity of polyphenols; *Potential candidates for anticancer flavonoids

Literature demonstrates the existence of relationship between the chemical structure of flavonoids and their anticancer properties[10,56,57]. For example, the anticancer activities of flavonoids depend on the number and position of hydroxyl groups, methoxy groups and the presence of C-C double bond in ring-B of the flavonoids[10,55].

A summary of the spectrum of biological activities of herbal extracts investigated in this study along with their polyphenol contents are presented in Table 6. In Table 6, the notation "Triple plus" is used to represent extremely high activity or large quantity of total polyphenols in the extracts. The notation "Double plus" is used to represent significant

activity or significant quantity of polyphenols. "Single plus" means average activity or average quantity of polyphenols in the extracts. It can be seen from Table 6 that the extracts from *A. quinata*, *A. officinarum*, *A. scoparia*, *L. chinensis*, *S. suberectus* and *A. rugosum* exhibited highly significant activities and also contain large quantities of polyphenols. It is therefore, concluded that these six herbs are extremely important candidates for the discovery of novel anticancer agents. Findings of this study strongly support the traditional use of many of these herbs. Especially, it is interesting to note that the six important herbs short listed by this research are extensively used by TCM practitioners in anticancer formulations[5,58].

CONCLUSION

Extremely good correlation was found between biological activities (antioxidant, anti-inflammatory and anticancer activities) and polyphenol contents of TCM herbs reported in this study. Six herbs (*A. quinata*, *A. officinarum*, *A. scoparia*, *L. chinensis*, *S. suberectus* and *A. rugosum*) are identified in this research as important candidates for the discovery of novel anticancer agents. It is interesting to note that, TCM practitioners extensively use all of these six herbs in anticancer formulations. The results presented in this study lead to the conclusion that corroboration of traditional knowledge with modern scientific tools has great potential to discover lead compounds for effective drugs.

SIGNIFICANCE STATEMENTS

This study used hot water extraction procedure to identify potential medicinal plants for the isolation of anticancer agents. Many cancer patients in developing countries cannot afford expensive chemotherapy treatment. Hence, the discovery of novel therapeutics from medicinal herbs is expected to provide tremendous benefit to the society. Plants identified in this study are suitable candidates for the discovery of compounds with significant anticancer properties that form potential leads to develop alternatives for the existing chemotherapeutic agents that are expensive with severe side effects.

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