

In vitro Anti-Proliferative Activity of the *Rubia tinctorum* and *Alkanna tinctoria* Root Extracts in Panel of Human Tumor Cell Lines

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Abstract

Cancer is a devastating disease and is considered number one killer worldwide. Herbal formulations had played a key role over the past several decades in the development of anti-cancer drugs. Medicinal plants, which are endemic in Jordan, are known for several biological activities in particular their anti-cancer activity. However, the anti-cancer efficacy of the root extracts of Jordanian *Rubia tinctorum* and *Alkanna tinctoria* is not yet reported. To address this issue, this study assessed the anti-cancer activity of some root extracts obtained from Jordanian *R. tinctorum* and *A. tinctoria* in different tumor cell lines including the tongue, bladder, colon, gastric, lungs, breast, pancreas, and renal tissue origins by modified propidium iodide (PI) based monolayer assay. Among the tested root extracts obtained by different solvent systems, *A. tinctoria* in 100 % ethanol and methanol showed prominent anti-cancer activity against MDA-MB-231 breast cancer cells (IC₅₀: 2.98 µg/ml, IC₇₀: 6.03 µg/ml), and CAL-27 tongue squamous carcinoma cells (IC₅₀: 3.86 µg/ml, IC₇₀: 5.97 µg/ml) respectively. Different solvent root extracts of *R. tinctorum* exhibited a similar trend of anti-tumor activity in both CAL-27 and MDA-MB-231 cells. The anti-proliferative property of the extracts on CAL-27 and MDA-MB-231 cells is unclear. However, it can be concluded that the observed anti-cancer potential can be attributed to the phenolic compounds of the extracts as high polar solvents were used for extraction. The current study forms the rationale for isolating significant amount of anti-cancer active compounds from *R. tinctorum* and *A. tinctoria*.

Key words: *Rubia tinctorum*, *Alkanna tinctoria*, Anti-cancer, CAL-27, MDA-MB-231

1. Introduction

Cancer is a major public health burden in both developed and developing countries. According to the American Cancer Society, deaths arising from cancer constitute 2-3 % of the annual deaths recorded worldwide. In the United States, about 600,920 Americans were expected to die of cancer in 2017, which translates to about 1650 people per day, and this is expected to be on the rise in 2018 (Siegel *et al.*, 2017). According to the morbidity and mortality rates associated with this disease, in addition to the critical economic burden, there are no effective strategies for the development of anti-cancer drugs. The emerging drug resistance concerning the existing cancer chemotherapy is another major hurdle to overcome in order to achieve therapeutic efficacy. Therefore, finding new drugs or drug formulations is highly warranted to alleviate the above-mentioned hurdles. Historically, plants have been the primary sources of natural products for drug discovery including plant-derived

agents, such as vinblastine (VBL) and vincristine (VCR), etoposide, paclitaxel (Taxol), docetaxel, topotecan, and irinotecan, which are amongst the most effective cancer chemotherapeutic agents (Cragg *et al.*, 2012).

In the Hashemite Kingdom of Jordan, natural sources consisting of more than 2500 wild plant species from 700 genera exist; of these, there are approximately 100 endemic species, 250 rare species, and 125 very rare species (Al-Eisawi *et al.*, 2000; Oran and Al Eisawi, 2014). Traditional medicine practices are part of the Jordanian culture, and there seems to be a wealth of ethnobotanical studies providing a new major contribution in the search for invaluable phyto-pharmaceuticals or the development of functional foods or nutraceuticals (Al-Khalil, 1995). Literature surveys based on published studies indicated that in Jordan and the neighboring countries, twenty-seven plant species are considered as traditional remedies for the treatment of different types of cancers (Hudaib *et al.*, 2008). Ethanolic extracts of more than seventy medicinal herbs from the Jordanian flora, belonging to sixty-seven species and thirty-four families,

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were evaluated for their anti-proliferative activity on a breast cancer cell line including *Alkanna* and *Rubia* genus (Abu-Dahab and Afifi, 2007). *Alkanna tinctoria* (Boraginaceae) and *Rubia tinctorum* (Rubiaceae) are widespread in the local regions of Jordan. Alkannin is a well-known phenolic compound present in *A. tinctoria* roots, and is reported for numerous biological activities, such as free radical scavenging (Kourounakis *et al.*, 2002), and anti-inflammatory properties (Kourounakis *et al.*, 2002). Alkannin has been reported to suppress the UV radiation induced apoptosis in human keratinocytes by modulating caspase and HSP70 signaling (Yoshihisa *et al.*, 2012). Naphthoquinones isolated from the root extract of *A. tinctoria* induces apoptosis and cell cycle arrest in colorectal cancer cells (Tung *et al.*, 2013). Assimopoulou *et al.* reported that hydroxynaphthoquinones and other metabolites have been isolated from ten species of the genus *Alkanna* (Assimopoulou *et al.*, 2006). The genus *Rubia* is known to be a source of several anthraquinones, and it is reported for different pharmacological activities such as anti-cancer, anti-microbial, anti-fungal, and anti-oxidant activities (Park *et al.*, 2009). The root extract of *R. tinctorum* is effective against kidney and bladder stones (Blomeke *et al.*, 1992; Westendorf *et al.*, 1998). The trihydroxy-anthraquinones alizarin and purpurin isolated from hairy root cultures of *R. tinctorum* inhibited the proliferation, adhesion and migration of melanoma cells (Eszter Lajko *et al.*, 2015).

A. tinctoria and *R. tinctorum* are well reported for anti-cancer activity. However, the anti-cancer efficacy of the root extract of Jordanian *A. tinctoria* and *R. tinctorum* is not yet reported. As phenolic compounds are known to be effective anti-cancer active principles, high polar solvents such as methanol, ethanol and water are always recommended for the extraction of such compounds more than low polar and non-polar solvents. In the current study, we used National Cancer Institute (NCI), USA recommended panel of tumor cell lines which are widely used to study anti-cancer activity of compounds (Heidi Ledford, 2016). In this study, roots of Jordanian *A. tinctoria* and *R. tinctorum* were extracted in different solvent systems (100 % ethanol, 50 % ethanol, 100 % methanol, hot water and cold water) and the extracts were screened in a panel of tumor cell lines (tongue, bladder, colon, gastric, lungs, breast, pancreas, and renal) for anti-cancer activity.

2. Materials and Methods

2.1. Plant Material

Rubia tinctorum roots were collected from the local regions of North Badia in Jordan (3489 kilometers from Amman). *Alkanna tinctoria* roots were collected from the local regions of Ajloun (72 kilometers from Amman) in Jordan. Both plants were taxonomically identified by the botanist based on anatomy and microscopic observation of internal structure of organs. Voucher specimen was deposited in the herbarium of the Biology Department of the Faculty of Science at the University of Jordan.

2.2. Solvent Extraction

The collected plant materials were shade-dried and finely powdered by a grinding mill. Both *R. tinctorum* and

A. tinctoria root powders were extracted with 100 % ethanol, 50 % ethanol, and 100 % methanol, and the extracts were centrifuged at a speed of 7000 rpm at 4°C for thirty minutes. Then extracts were lyophilized at -50°C and stored at 4°C until use for the experiment.

2.3. Hot Aqueous Extraction

R. tinctorum and *A. tinctoria* root powders were extracted with boiling water for thirty minutes, and the extracts were kept for cooling at room temperature for four hours under stirring. Then extracts were centrifuged at 5000 rpm at room temperature, filtered and lyophilized and stored at 4°C until use for the experiment.

2.4. Cell Lines

A Panel of tumor cell lines consists of tongue, bladder, colon, gastric, non-small cell lung, breast, pancreatic, and renal cancer. Non-PDX-derived cell lines were either kindly provided by the NCI (Bethesda, MD), or were purchased from ATCC (Rockville, MD) or DSMZ (Braunschweig, Germany), or JCRB (Japanese Collection of Research Biosources Cell Bank, Japan) (Table 1).

Table 1. Authenticated cell lines used for the study.

#	Cell line				STR Analysis
	Type	Name	Origin	STR Analysis	
1	Bladder	BXF RT112	DSMZ	Authentic 271	
2	Colon	CXF COLO205	NCI	Authentic	
3	Gastric	GXA MKN45	JCRB#0254	Ordered	
4	Tongue	HNXF CAL-27	DSMZ	Authentic	
5	Lung	LXFA 526L	Xenograft, Freiburg	Authentic 273	
6	Lung	LXFL 529L	Xenograft, Freiburg	Authentic	
7	Mammary	MAXF MDA-MB-231	ATCC	Authentic 274	
8	Mammary	MAXF SK-BR-3	ATCC, HTB-30	Authentic	
9	Pancreas	PAXF 1675L	Xenograft, Freiburg	Authentic 275	
10	Renal	RXF SN12C	NCI	Authentic	

JCRB: Japanese Collection of Research Bioresources ; ATCC: American Type Culture Collection, Rockville, MD, USA; NCI: National Cancer Institute, Bethesda, MD, USA; DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen Braunschweig, Germany

2.5. Cell Culture

Cell lines were routinely passaged once or twice weekly and maintained in culture for up to twenty passages. All cells were grown at 37°C in a humidified atmosphere with 5 % CO₂ in RPMI 1640 medium (25 mM HEPES, with L-glutamine, FG1385, Biochrom, Berlin, Germany) supplemented with 10 % (v/v) fetal calf serum (Sigma, Taufkirchen, Germany) and 0.1 mg/mL gentamicin (Life Technologies, Karlsruhe, Germany).

2.6. Anti-Cancer Assay

A modified propidium iodide (PI) based monolayer assay was used to assess the anti-cancer activity of the extracts (Dengler *et al.*, 1995). Briefly, cells were harvested from exponential phase cultures, counted and plated in ninety-six well flat-bottom microtiter plates at a cell density of 6,000 to 12,000 cells/well depending on the cell lines growth rate. After a twenty-four-hour recovery period, cells were allowed to resume exponential growth, 10 μL of culture medium (4 control wells/cell line/plate) or of culture medium with the extracts added. The extracts were applied at ten concentrations in half-log increments to 0.3 (v/v) or 100 $\mu\text{g mL}^{-1}$, and the treatment continued for four days. After four days of treatment, the cells were next washed with 200 μL PBS to remove the dead cells and debris, then 200 μL of a solution containing 7 $\mu\text{g/mL}$ propidium iodide (PI) and 0.1 % (v/v) Triton X-100 was added. After an incubation period of 1-2 hours at room temperature, fluorescence (FU) was measured using the Enspire Multimode Plate Reader (excitation $\lambda = 530$ nm, emission $\lambda = 620$ nm) to quantify the amount of attached viable cells.

2.7. Calculation of IC_{50} and IC_{70}

IC_{50} and IC_{70} values were calculated by four parameters non-linear curve fit using Oncotest Warehouse Software. For calculation of mean IC_{50} values, the geometric mean was used.

2.8. Data Evaluation

An assay was considered fully evaluable if all the following quality control criteria were fulfilled. Z'-factor calculated within the assay plate ≥ 0.5 (kevorokov and Makarenkov, 2005), Fluorescence intensity of > 500 U from the untreated control wells, equivalent to a

control/background ratio > 3.0 . and coefficient of variation in the growth control wells ≤ 30 %.

2.9. Sigmoidal Concentration Response Curve

Drug effects were expressed in terms of the percentage of the fluorescence signal, obtained by comparison of the mean signal in the treated wells with the mean signal of the untreated controls (expressed by the test-versus-control value, T/C-value [%]):

$$T/C (\%) = \frac{\text{mean fluorescence signal treated group}}{\text{mean fluorescence signal control group}} \times 100$$

Sigmoidal concentration-response curves were fitted to the data points obtained for each compound using four parameters non-linear curve fit (Oncotest Data Warehouse Software). IC values are reported as absolute and relative IC_{50} and absolute IC_{70} values. The absolute IC_{50} value reflects the concentration of the extracts that achieves T/C=50%. The absolute IC_{70} value gives the concentration of the extracts that achieves T/C=30%. The relative IC_{50} value is the concentration of extracts that gives a response half way between the top and bottom plateau of the sigmoidal concentration-response curve (inflection point of the curve).

3. Results

3.1. Geo Mean of Anti-Cancer Activity

The extracts were tested in half-log steps up to a test concentration of 100 $\mu\text{g/mL}$. Among the extracts tested, 100 % ethanol extract of *A. tinctoria* (geo mean IC_{50} value of 6.98 $\mu\text{g mL}^{-1}$) was most potent, followed by a 100 % methanol extract of *R. tinctorum* (Geo mean IC_{50} 10.66 $\mu\text{g mL}^{-1}$), 50 % ethanol extract of *R. tinctorum* (Geo mean IC_{50} 12.33 $\mu\text{g mL}^{-1}$) and 100 % methanol extract of *A. tinctoria* (Geo mean IC_{50} 14.91 $\mu\text{g mL}^{-1}$) (Table 2).

Table 2. The anti-cancer activity of *Alkanna tinctoria* and *Rubia tinctorum* root extracts (Geometric Mean value).

Cell line	<i>A. tinctoria</i> 100% ethanol extract ($\mu\text{g/mL}$)	<i>A. tinctoria</i> 100% methanol extract ($\mu\text{g/mL}$)	<i>R. tinctorum</i> 50% ethanol extract ($\mu\text{g/mL}$)	<i>R. tinctorum</i> 100% methanol extract ($\mu\text{g/mL}$)
BXFR T112	5	6.2	4.33	2.76
CXF COLO 205	10.57	33.33	26.93	23.4
GXA MKN45	11.45	14.1	10.44	8.33
HNXF CAL-27	3.83	4.61	2.94	2.53
LXFA 526	6.46			
LXFL 529	9.86	23.07	31.85	25.9
MAXF MDA-MB-231	3.2	10.55	5.14	5.51
MAXF SK-BR-3	8.53	19.99	28.23	25.03
PAXF 1657	6.4	18.87	13.06	14.51
RXF SN12C	10.67	29.53	30.55	30.55
Geo Mean IC_{50}	6.98	14.91	12.33	10.66

3.2. Anti-cancer Activity of *A. tinctoria*

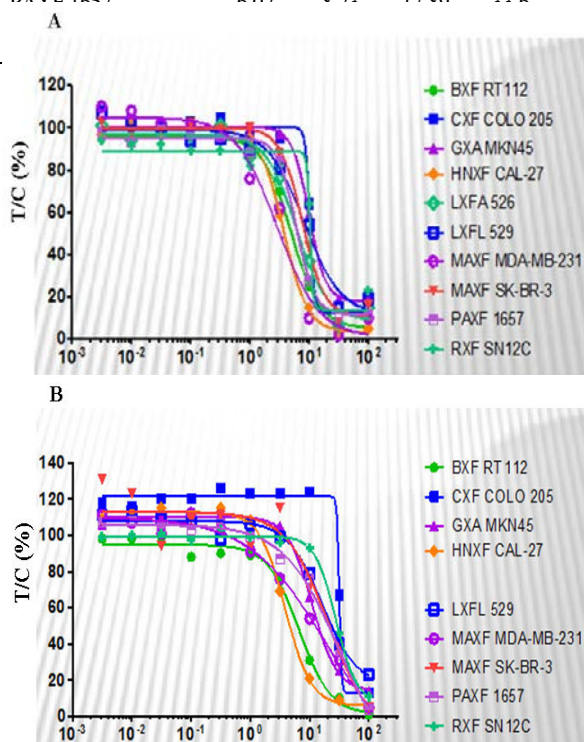
The 100 % ethanol extract of *A. tinctoria* exhibited the most potent *in vitro* anti-cancer activity against MDA-MB-231 cells (IC_{50} : 2.98 $\mu\text{g mL}^{-1}$, IC_{70} : 6.03 $\mu\text{g mL}^{-1}$) then on CAL-27 cells (IC_{50} : 3.75 $\mu\text{g mL}^{-1}$, IC_{70} : 5.59 $\mu\text{g mL}^{-1}$), whereas 100 % methanol extract of *A. tinctoria* showed more sensitivity towards CAL-27 cells (IC_{50} : 3.86 $\mu\text{g mL}^{-1}$

IC_{70} : 5.97 $\mu\text{g mL}^{-1}$) then on MDA-MB-231 cells (IC_{50} : 8.97 $\mu\text{g mL}^{-1}$, IC_{70} : 24.08 $\mu\text{g mL}^{-1}$) (Table 3). Both the 100 % ethanol and methanol extracts of *A. tinctoria* displayed dose-dependent decline in the cell survival of different tumor cells (Figure 1A and B), whereas, the 50 % ethanol and hot aqueous extract of *A. tinctoria* showed less anti-cancer activity (T/C: 29 % and 82 % respectively)

Table 3. The Minimum inhibitory concentration of the root extract of *Alkanna tinctoria* on a panel of tumor cell lines.

Figure 1. Dose response curve of anti-cancer efficacy of the root extract of *Alkanna tinctoria* on panel of tumor cell lines. A: *A. tinctoria* root extracted with 100 % ethanol; B: *A. tinctoria* root

Cell line	<i>A. tinctoria</i> 100% ethanol extract (µg/mL)		<i>A. tinctoria</i> 100% methanol extract (µg/mL)	
	IC ₅₀	IC ₇₀	IC ₅₀	IC ₇₀
BXF RT112	4.94	7.95	6.63	11.18
CXF COLO 205	10.31	11.08	31.62	33.84
GXA MKN45	9.87	13.05	11.10	16.79
HNXF CAL-27	3.75	5.59	3.86	5.97
LXFA 526	5.99	9.80		
LXFL 529	8.32	15.12	16.21	29.18
MAXF MDA-MB-231	2.98	6.03	8.97	24.08
MAXF SK-BR-3	7.70	11.15	17.23	31.28
PAXF 1657	6.02	9.22	17.90	22.6



extracted with 100 % methanol. Y axis indicates the percentage of survival. T/C: Test/Control.

3.3. Anti-Cancer Activity of *R. tinctorum*

The 100 % methanol extract of *R. tinctorum* was sensitize CAL-27 cells (IC₅₀: 2.30 µg mL⁻¹, IC₇₀: 3.36 µg mL⁻¹) followed on MDA-MB-231 cells (IC₅₀: 5.14 µg mL⁻¹, IC₇₀: 12.99 µg mL⁻¹) whereas, the 50 % ethanol extract of *R. tinctorum* sensitize CAL-27 cells (IC₅₀: 2.64 µg mL⁻¹, IC₇₀: 4.04 µg mL⁻¹) followed on MDA-MB-231 cells (IC₅₀: 5.68 µg mL⁻¹, IC₇₀: 12.37 µg mL⁻¹) (Table 4). Similar to *A. tinctoria* extracts, the 100 % methanol and 50 % ethanol extracts of *R. tinctorum* also showed a dose-dependent decline in the survival of tumor cells (Figure 2A and 2B).

Table 4. The Minimum inhibitory concentration of the root extract of *Rubia tinctorum* on a panel of tumor cell lines.

Cell line	<i>R. tinctorum</i> 50% ethanol extract (µg/mL)		<i>R. tinctorum</i> 100% methanol extract (µg/mL)	
	IC ₅₀	IC ₇₀	IC ₅₀	IC ₇₀
BXF RT112	5.02	9.63	3.17	5.38
CXF COLO 205	22.13	33.01	18.94	26.61
GXA MKN45	9.46	13.30	6.74	9.82
HNXF CAL-27	2.64	4.04	2.3	3.36
LXFA 526				
LXFL 529	31.44	81.82	19.19	4.19
MAXF MDA-MB-231	5.68	12.37	5.14	12.99
MAXF SK-BR-3	26.25	41.92	26.35	40.89
PAXF 1657	13.02	31.37	15.56	26.55
RXF SN12C	33.58	69.34	28.53	56.37

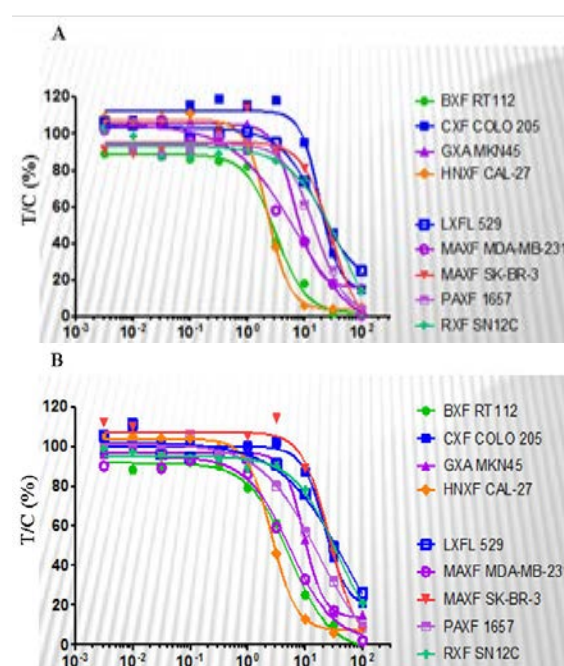


Figure 2. Dose response curve of anti-cancer efficacy of the root extract of *Rubia tinctorum* on a panel of tumor cell lines. A: *R. tinctorum* root extracted with 100 % methanol; B: *R. tinctorum* root extracted with 50 % ethanol. Y axis indicates the percentage of survival. T/C: Test/Control.

4. Discussion

It is well accepted that the plant constituents possess therapeutic and preventive activities against different cancer types. The Potential natural products have already contributed to 60 % of all anti-cancer drugs (Rates, 2001; Dorai and Aggarwal, 2004; Rabi and Bishayee, 2009). Various epidemiological and preclinical findings and the results of several early clinical studies convincingly argue for a definitive role of some selected dietary products in the treatment and prevention of cancers. Many of these agents target multiple signal transduction pathways, modulate cancer aneuploidy, tubulin binding, topoisomerases, and gene-specific targets, which vary widely depending on cancer origin (Pezzuto, 2008; Amin *et al.*, 2009; Nobili *et al.*, 2009). Optimizing the extraction

strategy to achieve a high yield of anti-cancer active principles is the paramount interest of cancer drug discovery researchers. Choosing an ideal solvent system and extraction stringency would pave the way for isolating a significant amount of active principles from natural resources (Zlotek *et al.*, 2016). In this study, different concentrations (50 % and 100 %) of ethanol, methanol and water (hot) were employed for the extraction. Both water and alcohols (ethanol and methanol) are well-known to extract polyphenolic compounds due to their high polar nature. *R. tinctorum* and *A. tinctoria* are well established for different biological activities such as antioxidant, anti-analgesic, anti-cancer etc. In particular, root extracts from *R. tinctorum* and *A. tinctoria* are reported for the presence of high amount of polyphenols alizarin and alkannin respectively (Tappeiner *et al.*, 2014; Eszter Lajko *et al.*, 2015). Unfortunately, *R. tinctorum* and *A. tinctoria* which are endemic in Jordan are not yet well-explored for any biological activities in particular, as an anti-cancer agent. To address this issue, we collected *R. tinctorum* and *A. tinctoria* root from local regions of Jordan and studied their anti-cancer activity in a panel of tumor cell lines (tongue, bladder, colon, gastric, lungs, breast, pancreas, and renal).

The extracts were tested at ten different concentrations in half-log dilution steps. Anti-tumor activity is expressed as absolute IC_{50} and IC_{70} values, calculated by non-linear regression analysis. Less than $5 \mu\text{g mL}^{-1}$ of 100 % methanol extract of *A. tinctoria* restricted the proliferation of MDA-MB-231 and CAL-27 cells significantly. In Jordan, the whole plant ethanolic extracts of *Alkanna strigosa* were previously tested for anti-cancer activity against MCF-7 cells, and it was reported that MCF-7 cells withstand the treatment and exhibit a 99 % survival (Abu-Dahab and Afifi, 2007). Interestingly, the plants belong to similar genus and endemic elicited difference in anti-cancer activities. This variation could be attributed to the difference in the presence of anti-cancer active principles. The presence of phenolic compounds such as alkannin, acetylalkannin, propionylalkannin, isobutylalkannin, angelylalkannin, β , β -dimethylacrylalkannin, isovalerylalkannin, α -methyl-n-butylalkannin, teracryl-alkannin, β - hydroxyisovalerylalkannin and naphtha-quinones in the root extract of *A. tinctoria* is well- reported (Assimopoulou *et al.*, 2006). Alkannin and angelylalkannin isolated from a 95 % ethanol root extract of *A. tinctoria* were shown to be effective anti-colon cancer agents (Tung *et al.*, 2013). In contrast, this study found that both CAL-27 and MDA-MB-231 cells are more sensitive than colon cancer cells (COLO205). Specific anti-proliferative property of 100 % ethanol and methanol root extracts of *A. tinctoria* against CAL-27 and MDA-MB-231 cells is not clear. High polar organic solvents ethanol and methanol are widely used and recommended to extract potential polyphenolic compounds (Tomsone *et al.*, 2012; Goncalves *et al.*, 2015). Synergistic mixture of phenolic compounds with the presence of solvents could achieve inter and intra molecular interaction and novel stereochemistry (Freeman *et al.*, 2010) and this may pave the way for specific anti-tumor potential towards CAL-27 and MDA-MB-231 cells. The chemical composition of 100 % ethanol and 100 % methanol extracts of *A. tinctoria* root should be studied in detail to address this issue further.

Mazzio *et al.* reported that the ethanolic root extract of *R. tinctorum* can restrict the growth of MDA-MB-231 cells significantly with an IC_{50} value of $20.5 \mu\text{g mL}^{-1}$ (Mazzio *et al.*, 2014). In the current study, it was found that both the 50 % ethanol ($5.14 \mu\text{g mL}^{-1}$) and the 100 % methanol ($5.68 \mu\text{g mL}^{-1}$) extracts of *R. tinctorum* reduced the growth of MDA-MB-231 cells with a less concentration than reported before. Noticeably, the 50 % ethanol extract of *R. tinctorum* showed a better anti-cancer activity than the 100 % methanol extract. Mixing water with alcohol could enhance the polarity further, thus, aqueous alcoholic extract could extract a high amount of phenolic compounds than absolute solvent extracts. Sultana *et al.* reported that aqueous alcoholic extraction can achieve a high yield of antioxidant phenolic compounds than absolute solvent extraction (Sultana *et al.*, 2009). The root extract of *Rubia cordifolia* L contains a rich amount of the phenolic compound mollugin inhibiting the proliferation of HeLa cells by modulating TNF- α and NF- κ B signaling (Zhe Wang *et al.*, 2017). Roots of *Rubia yunnanensis* contain triterpenoid rubiarbonol G induced apoptosis and cell cycle arrest in HeLa cells (Zeng *et al.*, 2017). *R. tinctorum* is known to accumulate a rich amount of phenolic compounds such as alizarin, purpurin etc. (Eszter Lajko *et al.*, 2015). Hydroxyl group of phenolic compounds can intercalate with DNA and induce irreparable DNA damage and apoptosis. Water extraction can achieve a high yield of phenolic compounds compared to organic solvent due to its high polarity; however, it depends on the nature of plants and its parts. Naturally, the roots are the harder part of plant compared to the leaves and stem. The aqueous extract is widely considered to be safe for oral ingestion for the various ailments; however, the efficient extraction of active principles from plant parts depends on the vulnerability of cellular architecture of plant parts. In this study, it was found that the root extracts obtained from *A. tinctoria* and *R. tinctorum* using alcohol can sensitize the tumor cells more significantly than the aqueous extract. It clearly indicates that to extract the active principles from the roots of *A. tinctoria* and *R. tinctorum* requires organic solvents due to their harder nature, for which water may not be sufficient to destruct the cellular architecture to achieve a high yield of the phenolic compounds.

5. Conclusion

The results of the present investigation revealed that the root extracts of *R. tinctorum* were found to be more potent anti-cancer agents compared to the *A. tinctoria* root extracts. It is recommended that the chemical composition of the *R. tinctorum* root extracts should be studied in detail. Studies on the efficacy of root extracts on apoptosis (intrinsic and extrinsic) and on cell survival signaling molecules should be done to find out molecular mechanism. Further research is needed to assess the anti-cancer effect of root extracts' derived compounds *in vivo* model and to develop cancer drug formulation from *R. tinctorum*.

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