Investigating the antiangiogenic potential of *Rumex vesicarius* (humeidh), anticancer activity in cancer cell lines and assessment of developmental toxicity in zebrafish embryos

Muhammad Farooq a,⇑, Nael Abutaha a, Shahid Mahboob b, Almohannad Baabbada a, Nawaf D. Almoutiri a, Mohammad Ahmed A.M. Wadaana a

*a College of Science, Department of Zoology, King Saud University, 11451 Riyadh, Saudi Arabia
b Department of Zoology, College of Science, King Saud University, 11451 Riyadh, Saudi Arabia

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**A B S T R A C T**

Recent trends in anticancer therapy is to use therapeutic agents which not only kill the cancer cell, but are less toxic to surrounding normal cells/tissue. One approach is to cut the nutrient supply to growing tumor cells, by blocking the formation of new blood vessels around the tumor. As the phytochemicals and botanical crude extracts have proven their efficacy as natural antiangiogenic agents with minimum toxicities, there is need to explore varieties of medicinal plants for novel antiangiogenic compounds.

*Rumex vesicarius* L. (Humeidh), is an annual herbal plant with proven medicinal values. The antiangiogenic potential, and developmental toxicity of humeidh in experimental animal models has never been studied before. The crude extracts were prepared from the roots, stems, leaves and flowers of *Rumex vesicarius* L. in methanol, chloroform, ethyl acetate and n-hexane. The developmental toxicity screening in zebrafish embryos, has revealed that *Rumex vesicarius* was not toxic to zebrafish embryos. The chloroform stem extract showed significant level of antiangiogenic activity in zebrafish angiogenic assay on a dose dependent manner. Thirty five (35) bioactive compounds were identified by gas chromatography mass spectrophotometry (GC–MS) analysis in the stem extract of *Rumex vesicarius*. Propanoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester, Butane, 1,2,3-tris(trimethylsiloxy), and Butanedioic acid, bis (trimethylsilyl) ester were identified as major compound present in the stem of *R. vasicarius*.

The anticancer activity of roots, stem, leaves and flowers crude extract was evaluated in human breast cancer (MCF7), human colon carcinoma (Lovo, and Caco-2), human hepatocellular carcinoma (HepG2) cell lines. Most of the crude extracts did not show significant level of cytotoxicity in tested cancer cells line, except, chloroform extract of stem which exhibited strong anticancer activity in all tested cancer cells with IC50 values in micro molar range.

Based on these results, it is recommended that formulation prepared from *R. vesicarius* can further be tested in clinical trials in order to explore its therapeutic potential as an effective and safe natural anticancer product.

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1. Introduction

Solid tumors produce blood vessels to get nourish and to migrate to other organs, a process known as metastasis. The angiogenesis (formation of secondary blood vessels) is a normal process during embryonic development, and during wound healing and the menstrual cycle. However, angiogenesis get activated in pathological condition, such as in cancer. The activation of angiogenesis exclusively by cancer, and quiescence in normal cells makes regulation of angiogenesis as an attractive therapeutic target for anti-tumor drug discovery* (Al-Abd et al., 2017)*, and hence many small molecules have been synthesized and tried in tumor cells to suppress the angiogenesis (Khalid et al., 2016; Wang et al., 2015), however, majority of these compounds either failed in clinical trials, or discontinued due to having lot of side effects (Cao, 2016; Lu et al., 2019; Medina et al., 2007). One reason for the inefficacy of
angiogenesis inhibitors could be due to the fact that, the majority of synthetic anti-angiogenic molecules target only single angiogenic pathway for example, interacting only to "vascular endothelial growth factor (VEGF)" or its receptors, VEGF is a protein which is responsible for the proliferation of endothelial cells (Abdel-Qadir et al., 2017; Qin et al., 2019). Crude extract or pure compounds derived from traditional medicinal plants act on multiple targets and have shown good anti-angiogenic effects with least toxicities (Erices et al., 2018; Gezici and Sekeroglu, 2019; Song et al., 2019). Hence, there is a need to explore new antiangiogenic natural sources most likely from medicinal plants and herbs by suitable in vivo and in vitro angiogenic assays.

In order to find out novel natural antiangiogenic products, the Saudi medicinal plants were explored in this study. One hundred and fifty (150) medicinal plants were collected from various regions of Saudi Arabia and from folk medicine practitioners (aatar). The crude extracts were prepared in methanol, chloroform, ethyl acetate and hexane. The antiangiogenic activity was evaluated in zebrafish transgenic line which express green fluorescent protein constitutively in blood vessels (Lawson and Weinstein, 2002).

"Rumex vesicarius" bladder dock (Arabic; Humeidh) has been identified as one of the plant, which had showed significant level of antiangiogenic activity in our pilot study. *R. vesicarius* is a branched tender perennial herbal plant which belongs to Polygonaceae family and is widely distributed throughout Saudi Arabia (Harley, 1991). *R. vesicarius* has been used in traditional medicine as flattulence, tonic, digestion enhancer, laxative, Anti-nausea, spleen disorders, antiasthma, bronchitis, analgesic and in some hepatic diseases in Egypt, India, and Saudi Arabia (Vasas et al., 2015).

The antiangiogenic property of *R. vesicarius* has not been reported before, and hence, the antiangiogenic activity and developmental toxicity of *R. vesicarius* was explored in zebrafish embryos in this study. The bioactive compounds present in *R. vesicarius* were identified using Gas chromatography-Mass spectrometry (GC-MS) analysis. In order to find out “target protein” for the major bioactive compounds present in the stem of *R. vesicarius* an online proteome web tool “Swiss target prediction” was used. (Gfeller et al., 2013). The anti-cancer activity of the crude extracts of roots, stem, and flowers of *R. vesicarius* was checked in human breast cancer (MCF7), human colon carcinoma (Lovo, and Caco-2), and human hepatocellular carcinoma (HepG2) cell lines.

2. Material and methods

2.1. Plant collection and preparation of crude extract

The plant was collected in flowering season (February–March) from Riyadh region, Saudi Arabia. The plant was washed thoroughly with running tap water. Roots, stems, leaves and flower were separated and let to dry under shade for several days. The crude extract from the roots, stem, leaves and flower was prepared in methanol, chloroform, hexane, and ethyl acetate essentially same as reported previously (Nasr et al., 2018).

2.2. Cytotoxicity evaluation

The toxicity of roots, stem, leaves, and flowers of *R. vesicarius* was tested in human breast cancer cell line (MCF7), human colon cancer cell lines (Lovo, Caco-2), and human liver carcinoma cell lines (HepG2). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric proliferation assay was used to assess the cytotoxicity of the extracts on cancer cell lines using Vybrant® MTT Cell Proliferation Assay Kit (Cat # V13154 lot # 1,129,031 Invitrogen) following the protocol provided by the manufacturer. The cell culture conditions were same as reported previously (Farooq et al., 2019).

2.3. Zebrafish developmental toxicity and angiogenic assays

The source of animals and maintenance was the same as described previously (Farooq et al., 2018). Thirty (30) embryos were exposed to extracts at gastrulation stage with serial dilution (10–500 µM) of each extract. The effect of extracts on embryonic survival, toxicity was monitored in wild type embryos. The treated embryos were examined at 24, 48, and 72 h post exposure. The developmental toxicity was evaluated based on, 1) Calculating the % lethality in response to serial dilution of the extracts, 2) embryonic abnormalities by observing the defects in otoliths, eyes, somite formation, tail detachment, heartbeat, blood circulation, hatching, and active swimming and % embryonic defects in each condition was calculated by equation

\[
\text{Number of embryos with defects/total number of embryos} \times 100
\]

The antiangiogenic activity was evaluated in transgenic zebrafish embryos. The experiments were repeated for three times. The percent antiangiogenic activity in transgenic zebrafish embryos was assessed by the equation as follow.

\[
\frac{\text{Average } \% \text{ of isv-siv in treated embryos}}{\text{Average } \% \text{ of isv-siv in control embryos}} \times 100
\]

The blood vessels in control and treated live Tg (flil1:EGFP) were counted by observing the embryos using fluorescent microscope (Zeiss Observer D1; Zeiss, Germany) under FITC filter. At least five embryos in each condition were examined in order to calculate the mean % antiangiogenic activity of all the extracts.

3. Gas chromatography-mass spectrometry analysis

3.1. Extraction using ultra-sonication

Plant samples (1 g) was added to 25 mL of ethyl acetate: hexane solvent mixture (1:1) in a beaker. The ultrasonic probe was placed in the beaker and power was set to 60 W. Ultrasonic temperature was maintained at 45 °C by using a water bath without any external heating. After extraction, the sample was filtered using polypropylene sheet membrane (157 µm thickness and 0.2 µm pore size) was purchased from Membrana (Wuppertal, Germany). Extract contains both polar and non-polar analytes, hence the extract was derivatized using Bis(trimethylsilyl)trifluoroacetamide (BSTFA). A ratio of extract and BSTFA (1:1) was added and then the 1 µL of extract was injected to GC–MS. Shimadzu (Kyoto, Japan) QP2010 GC–MS instrument equipped with a Shimadzu AOC-20s auto sampler and AOC-20 auto injector and Rxi-5 SI MS column with thickness of 0.25 µm, length of 30.0 m and diameter of 0.25 mm (Restek, Bellefonte, US). The high purity helium gas was employed as carrier gas at flow rate of 1.01 mL/min. All the samples were injected in split mode (1:100 ratio) and GC injection port temperature was kept 250 °C. The opening time of split vent was 1.0 min. The GC–MS interface temperature was 220 °C and ion source temperature was 200 °C. The oven temperature was programmed as follows: initial temperature was 40 °C and held for 1 min; then increased to 100 °C at 10°C/min and held for 2 min; then increased to 165 °C at 10 °C/min and held for 0 min; then increased to 190 °C at 6 °C/min and held for 3 min; after that it was increased to 220 °C at 3 °C/min and held for 3 min; and finally
increased to 240 °C at 2 °C/min and held for 1 min. For qualitative analysis, data acquisition was performed in scan mode to confirm the retention times of target compounds. The identification of the compounds was performed by similarity searches and mass spectra data in the NIST (National Institute for Standard and Technology) MS Library.

3.2. Statistical analysis and calculation of IC<sub>50</sub> and LC<sub>50</sub>

The data of zebrafish mortalities to serial dilution of the extract was put in Excel sheet and mean values, standard deviation were calculated. Similarly the dose response to cancer cells to the extract was obtained. The IC<sub>50</sub> values for the cytotoxicity in cancer cell lines, and LD<sub>50</sub> values for zebrafish mortality were calculated by probit analysis (R.H.D, 1952).

4. Results

4.1. Developmental toxicity R. vesicarius in zebrafish embryos

The comparative developmental toxicity data at 24, 48 and 72 hpf of chloroform fraction of stem part is shown in Table 1. It is quite evident from these values that chloroform extract of stem part of R. vesicarius did not induce any significant level of toxicity in zebrafish embryos. Similarly no developmental toxicity was observed in zebrafish embryos treated with crude extract prepared in hexane, methanol, chloroform and water from the stem part of R. vesicarius. Zebrafish embryos treated with crude extracts prepared from root, leaves, and flowers did not show any significant level of toxicity as well.

4.2. Chloroform stem extract of R. vesicarius exhibited antiangiogenic activity in zebrafish embryos.

The percent antiangiogenic activity of crude extract in various solvent of roots, stem, leaves and flowers R. vesicarius is presented in Table 2. The antiangiogenic activity was observed with chloroform crude extract prepared from the stem part of R. vesicarius (Fig. 1). The stem CHCl<sub>3</sub> extract obstructed ≥70% of inter-segmental blood vessels (isv) and 100% of sub-intestinal vein (siv) blood vessels formation in treated embryos at 30 μg/ml (Fig. 1B and C). The crude extracts from the stem part prepared in methanol, hexane, chloroform and water showed very weak (<10%) level of antiangiogenic activity, which was statistically insignificant compared to untreated control embryos. Moreover, the crude extract prepared from other plants parts such as roots, leaves and flowers of R. vesicarius were completely active in term of antiangiogenic activity in transgenic zebrafish embryos.

Semaxanib (SU 5416) was used as positive control. As expected Semaxanib has shown potent antiangiogenic activity in zebrafish embryos. As shown in Fig. 1, the zebrafish embryos treated with Semaxanib (1 μM) exhibited malformation of the inter-segmental and sub-intestinal angiogenic blood vessels. However, Semaxanib treated embryos were smaller in size as compared to untreated control and also to embryos which were treated with various solvents extract of R. vesicarius, were unable to hatch, had blood pooling of blood in trunk region, lacked pigmentation, and had abnormal craniofacial structure 1 μM concentration.

4.3. The stem crude extract of R. vesicarius affected the cell survival of human cancer cell lines

Four different human cancer cell lines were used in order to judge the anticancer activity of R. vesicarius. The cytotoxicity of roots, stem, leaves, and flowers of R. vesicarius is expressed by IC<sub>50</sub> values which is presented in Table 2. It is quite apparent from the data in Table 2 that the crude extracts from roots, stem and leaves has shown cytotoxicity in tested cancer cells, but flower part was completely inactive. Among all the extracts, the chloroform extract of stem was most active with IC<sub>50</sub> values of 33.45 ± 0.24, 35.90 ± 0.33, 45.22 ± 0.24, and 62.56 ± 0.02 μM in MCF7, Lovo, Caco-2 and HepG2 cells respectively. The second level of cytotoxicity was shown by ethyl acetate extract of stem with IC<sub>50</sub> values of 64.26 ± 0.33, 78.32 ± 0.21, 86.35 ± 0.01, and 159 ± 0.32 μM in MCF7, Lovo, Caco-2 and HepG2 cells. The IC<sub>50</sub> values of the crude extracts prepared from the roots, and leaves were above than 500 μM.

| Table 1 | The developmental toxicity of Rumex vesicarius in zebrafish embryos. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Rumex vesicarius stem | Embryo lethality¥ | Otoliths | Eyes | Heart beat | Blood circulation | Hatching | Active swimming |
| | Mean* | S.D | Mean | S.D | Mean | S.D | Mean | S.D | Mean* | S.D | Mean | S.D |
| 24hpf | Control | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 |
| | 0.001 | 1 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 |
| | 0.01 | 1 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 |
| | 1 | 1 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 |
| | 10 | 2 | ±0.57 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 |
| | 100 | 2 | ±0.57 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 |
| | 300 | 5 | ±0.47 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 |
| 48hpf | Control | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 |
| | 0.001 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 |
| | 0.01 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 |
| | 1 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 |
| | 10 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 |
| | 100 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 |
| | 300 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 |
| 72hpf | Control | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 |
| | 0.001 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 |
| | 0.01 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 |
| | 1 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 |
| | 10 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 |
| | 100 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 |
| | 300 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 | 0 | ±0.0 |

¥ Percentage of embryos with developmental defects.* mean values from three biological replicates. S.D; standard deviation.
4.4. Identification of potential antiangiogenic molecule in the stem of *R. vesicarius*

Table 3 shows the GC–MS spectrum and the list of compounds with molecular weight, retention time, and percentage of the compound present in extract with molecule formula. The GC–MS spectrum showed thirty five peaks corresponding to different compounds. Most of the compounds are novel and were identified in the stem of *R. vesicarius* for the first time in this study. The “Propanoic acid, 2,3-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester, Butanediolic acid, bis[(trimethylsilyl) ester] and Butane, 1,2,3-tris (trimethylsiloxy) were identified as major compounds.

In order to find out the proteins target to which these molecule will bind, “Swiss target prediction” was used. Supplementary Tables 1–3 contain the list of all the possible protein targets for major compounds identified in the stem of *R. vesicarius*. Among all the proteins, Leucine-rich repeat serine/threonine protein kinase 2 (LRRK2), Adenosine receptors (A1, A2a, A2b, and A3), FK506 binding protein 1A (FKBPIA), Tyrosine-protein kinase JAK3, JAK1, and JAK2, Muscarinic acetylcholine receptor, Glycogen synthase kinase-3 beta and alpha, Vascular Endothelial receptor 2 (VDR) and craniofacial cartilage deformities, at 1 μM concentration and more than 70% mortalities at 5 μM or more concentration. The herbs based antiangiogenic phytochemicals include, “Artemisinin”, *Viscum album* (Harmsma et al., 2004), *Curcuma longa* (Arbiser et al., 1998), *Scutellaria baicalensis* (Chinese Skullcap) (Liu et al., 2003), Resveratrol and Proanthocyanidin (Grape Seed Extract) (Cao et al., 2005). As the phytochemicals and botanical crude extracts have proven their efficacy as natural antiangiogenic agents with minimum toxicities (Dhillon et al., 2008; Ishikawa et al., 2006), there is urgent need to explore new medicinal plants in order to identify and isolate novel antiangiogenic compounds.

The chloroform extract of stem part was subjected to phytochemical analysis using GC–MS in order to identify antiangiogenic compounds. The major compounds which were identified were 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester, Butane, 1,2,3-tris (trimethylsiloxy), and Butanediolic acid, bis[(trimethylsilyl) ester]. The online Swiss target prediction tool was used to find out the protein target for these molecules. Interestingly, the target proteins were all angio genesis regulator proteins and were reported to be involve in normal or pathological angiogenesis. The predicted proteins target for the major compounds and their relationship to angiogenesis are shown in Table 4. These bioactive molecules need to be isolated in pure form from *R. vesicarius* and then should be used as bioactivity guided experimental animal model. Zebrafish embryos were used as bioactivity guided experimental animal model. Zebrafish transgenic line Tg (flh1:EGFP) which expresses enhanced green fluorescent protein (EGFP) in endothelial cells (blood vessels) provides an excellent in vivo animal model to screen angiogenic compounds (Butler et al., 2017; Raghunath et al., 2009).

The chloroform extract of stem of *R. vesicarius* suppressed the formation of angiogenic blood vessels in zebrafish embryos. The stem CHCl3 extract obstructed >70% of inter-somatic blood vessels (isv) and 100% of sub-intestinal vein (siv) blood vessels formation in treated embryos at 30 μg/ml. The *R. vesicarius* did not produce any embryonic abnormalities or toxicity at this concentration and even at very high tested concentration of 700 μM in zebrafish embryos. This mean that inhibition of blood vessels as seen in zebrafish embryos by *R. vesicarius* is the primary biological activity and it is not due to the secondary effect by toxicity.

Semanaxib (SU 5416) which is an inhibitor of the vascular endothelial growth factor receptor (Fong et al., 1999; Haspel et al., 2002; Mendel et al., 2000) was used as positive control. However, Semanaxib induced severe abnormalities and toxicity in zebrafish embryos besides inhibiting the angiogenic blood vessels. These abnormalities were shortened bodies, en large cardiac edema, cardiac hypertrophy, pooling of blood cells (hemorrhages), and craniofacial cartilage deformities, at ≤1 μM concentration and more than 70% mortalities at 5 μM or more concentration.

5. Discussion

Angiogenesis intervention is considered as one of the target for anticancer therapy due to the concept that the tumor growth can be control if oxygen and nutrients supply to the tumor site could be reduced (Viallard and Lariviere, 2017).

Saudi Arabian flora is composed of over two thousand species comprising of more than one hundred forty two families which contain not only quite big number of indigenous species, but also have mixture of Asian, African and Mediterranean regions (Rahman et al., 2004). However, the antiangiogenic potential of Saudi medicinal plants is least explored, and hence efforts are needed to screen these plants through suitable antiangiogenic assays to find out novel antiangiogenic phytochemicals.

A bioactivity guided identification of antiangiogenic medicinal plants strategy was adopted in this study. Zebrafish embryos were used as bioactivity guided experimental animal model. Zebrafish transgenic line Tg (flh1:EGFP) which expresses enhanced green fluorescence protein (EGFP) in endothelial cells (blood vessels) provides an excellent in vivo animal model to screen angiogenic compounds (Butler et al., 2017; Raghunath et al., 2009).

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The chloroform extract of stem part was subjected to phytochemical analysis using GC–MS in order to identify antiangiogenic compounds. The major compounds which were identified were 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester, Butane, 1,2,3-tris (trimethylsiloxy), and Butanediolic acid, bis[(trimethylsilyl) ester]. The online Swiss target prediction tool was used to find out the protein target for these molecules. Interestingly, the target proteins were all angiogenesis regulator proteins and were reported to be involve in normal or pathological angiogenesis. The predicted proteins target for the major compounds and their relationship to angiogenesis are shown in Table 4. These bioactive molecules need to be isolated in pure form from *R. vesicarius* and then should be

| Cell line | Part of the plants used | Cytotoxicity* (IC_{50}) (μg/mL) | n-hexane | Chloroform | Ethyl acetate | Methanol | Water |
|-----------|------------------------|---------------------------------|---------|------------|--------------|---------|-------|
| MCF7      | Roots                  | N.A                             | N.A     | N.A        | N.A          | N.A     | N.A   |
|           | Stem                   | ≥300                            | 33.45 ± 0.24 | 64.26 ± 0.33 | ≥300       | N.A     | N.A   |
|           | Leaves                 | ≥300                            | 500     | ≥500       | N.A          | N.A     | N.A   |
|           | flower                 | N.A                             | N.A     | N.A        | N.A          | N.A     | N.A   |
| Lovo      | Roots                  | N.A                             | N.A     | N.A        | N.A          | N.A     | N.A   |
|           | Stem                   | ≥300                            | 35.90 ± 0.33 | 78.32 ± 0.21 | ≥300       | N.A     | N.A   |
|           | Leaves                 | ≥300                            | 164 ± 0.24 | ≥300       | ≥300         | N.A     | N.A   |
|           | flower                 | N.A                             | N.A     | N.A        | N.A          | N.A     | N.A   |
| Caco-2    | Roots                  | N.A                             | N.A     | N.A        | N.A          | N.A     | N.A   |
|           | Stem                   | ≥300                            | 45.22 ± 0.24 | 86.35 ± 0.01 | N.A        | N.A     | N.A   |
|           | Leaves                 | ≥300                            | ≥300   | N.A       | N.A          | N.A     | N.A   |
|           | flower                 | N.A                             | N.A     | N.A        | N.A          | N.A     | N.A   |
| HepG2     | Roots                  | N.A                             | N.A     | N.A        | N.A          | N.A     | N.A   |
|           | Stem                   | ≥500                            | 235.56 ± 0.02 | 159 ± 0.32   | ≥500       | N.A     | N.A   |
|           | Leaves                 | ≥500                            | ≥500   | ≥500       | N.A          | N.A     | N.A   |
|           | flower                 | N.A                             | N.A     | N.A        | N.A          | N.A     | N.A   |

* The values are average of triplicate experiments, N.A: Not active.
tested in suitable antiangiogenic assays, however, the antiangiogenic activity of stem of *R. vesicarius* as observed in this study, could be due to the synergetic effect of bioactive molecules which are present in the crude methanol extract of stem.

The MTT cell proliferation assays has revealed that the crude extracts from the roots, leaves, and flowers did not show cytotoxicity towards tested cancer cells lines. Similarly the methanol, hexane extract prepared from the stem part of the plant showed weaker cytotoxicity and with IC\textsubscript{50} values were more than 300 \textmu M. The chloroform and ethyl acetate extract from the stem part *R. vesicarius* has induced significant level of cytotoxicity in MCF7, HepG2, Lovo, and Caco-2 cell lines with IC\textsubscript{50} values less than 50 \textmu M. The anticancer activity of *R. vesicarius* in various human cancer cell lines has also been reported by other studies. The methanol extract prepared from the *R. vesicarius* inhibited the proliferation of CCRF-CEM (human T lymphoblastoid) cells, with IC\textsubscript{50} value of 37.13 \textmu g/mL (Kuete et al., 2013), whole plant methanol extract showed cytotoxicity against HepG2 cells (human hepatocellular carcinoma) with IC\textsubscript{50} values 563.33 \pm 0.8. An Ethyl acetate extract of *R. vesicarius* prepared from leaves has shown cytotoxicity in HT-29 (human colorectal carcinoma cell line) and PC-3 (human prostate cancer) with IC\textsubscript{50} values 54.81 \pm 0.8405 70.90 \pm 1.3080 respectively (Manure, 2017). The anticancer profile of various parts of *R. vesicarius* in human breast carcinoma cell line (MDA-MB-231) have been previously reported by us (Nasr et al., 2018). The IC\textsubscript{50} values of chloroform and ethyl acetate extract prepared from the stem of *R. vesicarius* in this study, greatly varies with published results. It could be due to the climatic differences from where the plant has been collected as the chemical nature of soil and nutrients greatly affect the biological activity of plants. Moreover, the anticancer activity of this plant has never been reported in MCF7, Caco-2 and lovo. The IC\textsubscript{50} values in Caco-2 and Lovo which are derived from human colon cancer are closely related to anticancer activity of *R. vesicarius* in HT-29 cells which are also derived from human colon cancer. The polarity of the solvent used to prepare the extract also contribute a lot for the variation in biological activity.

Zebrafish screening assays also provided an insight into the development toxicity of *R. vesicarius* in animal system. The acute and chronic toxicities studies have reported that *R. vesicarius* was...
Table 3
GC–MS analysis of the stem of *Rumex vesicarius*.

| No | Retention time | Percentage | Compound name and structure |
|----|----------------|------------|-----------------------------|
| 1  | 6.178          | 4.64       | Nicotinaldehyde thiosemicarbazone tritms C₁₆H₃₂N₄SSi₃ |
| 2  | 6.216          | 3.63       | 2-Oxovaleric acid, tert-butyldimethylsilyl ester C₁₁H₂₂O₃Si |
| 3  | 6.435          | 9.65       | Propanoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester C₉H₂₂O₃Si₂ |
| 4  | 6.688          | 2.51       | Acetic acid, [(trimethylsilyl)oxy]-, trimethylsilyl ester C₈H₂₀O₃Si₂ |
| 5  | 6.78           | 1.05       | Sulforidazine C₂₁H₂₆N₂O₂S₂ |
| 6  | 6.816          | 5.06       | 2-Cyclopenten-1-one, 2,3-dimethyl- C₇H₁₀O |
| 7  | 6.866          | 3.6        | D-Mannitol C₁₂H₂₂O₆ |
| 8  | 7.494          | 2.05       | Trimethylsilyl ether of glycerol C₁₂H₃₂O₃Si₃ |
Table 3 (continued)

| No | Retention time | PECENTAGE | Compound name | Formula | structure |
|----|----------------|-----------|---------------|---------|-----------|
| 9  | 7.68           | 0.69      | 3,4-Dihydroxymandelic acid, ethyl ester, tri-TMS | C19H36O5Si3 |
| 10 | 7.737          | 2.17      | Oxanilic acid, O,O'-bis(trimethylsilyl) | C14H23NO3Si2 |
| 11 | 8.16           | 1.15      | Pentanoic acid, trimethylsilyl ester | CBH18O2Si |
| 12 | 8.245          | 7.5       | Butane, 1,2,3-tris(trimethylsiloxy)- | C13H34O3Si3 |
| 13 | 8.462          | 1.2       | Propanoic acid, | C10H24O3Si2 |
| 14 | 8.874          | 2.47      | Butanoic acid, | C13H32O4Si3 |
| 15 | 9.031          | 0.66      | 1H-Indole-2-carboxylic acid, 5-ethyl-1-(trimethylsilyl)-, trimethylsilyl ester | C17H27NO2Si2 |
| 16 | 9.541          | 4.59      | Trimethylsilyl ether of glycerol | Trimethylsilyl ether of glycerol |

(continued on next page)
| No | Retention time | Percentage | Compound name | Formula | Structure |
|----|----------------|------------|---------------|---------|-----------|
| 17 | 9.624          | 0.54       | Monoamidomalonic acid, tris(trimethylsilyl) | C12H29NO3Si3 |
| 18 | 10.013         | 1.37       | 3-Octenoic acid, trimethylsilyl ester | 3-Octenoic acid, trimethylsilyl ester |
| 19 | 10.06          | 0.71       | Isotridecanol- | C13H28O |
| 20 | 10.146         | 7.76       | Butanedioic acid, bis(trimethylsilyl) ester | C10H22O4Si2 |
| 21 | 10.324         | 3.21       | Propanoic acid, 2,3-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester | C12H30O4Si3 |
| 22 | 10.819         | 0.67       | Nonanoic acid, trimethylsilyl ester | C12H26O2Si |
| 23 | 10.927         | 0.7        | 2,3-Dimethyl-3-hydroxyglutaric acid, tris(trimethylsilyl) | C16H36O5Si3 |
| 24 | 11.417         | 0.92       | Tetradecane | C14H30 |
| 25 | 12.429         | 4.13       | Malic acid, O-(trimethylsilyl)-, bis(trimethylsilyl)ester | C13H30O5Si3 |
Table 3 (continued)

| No | Retention time | Percentage | Compound name | Formula | Structure |
|----|----------------|------------|---------------|---------|-----------|
| 26 | 13.125         | 1.08       | 3-Octadecanone | C18H36O | ![Structure](image1) |
| 27 | 13.565         | 0.85       | 2-Hydroxyisocaproic acid, trimethylsilyl ester | C9H20O3Si | ![Structure](image2) |
| 28 | 14.133         | 1.34       | D-Ribofuranose, 1,2,3,5-tetrakis-O-(trimethylsilyl)- | C17H42O5Si4 | ![Structure](image3) |
| 39 | 15.719         | 1.08       | 2-Deoxy-3,4,5-tris-O-(trimethylsilyl)pentose | C14H34O4Si3 | ![Structure](image4) |
| 30 | 18.194         | 5.68       | D-Xylofuranose, | C17H42O5Si4 | ![Structure](image5) |
| 31 | 18.975         | 3.07       | 3,7,11,15-Tetramethyl-2-hexadecen-1-ol | C20H40O | ![Structure](image6) |
| 32 | 19.099         | 5.59       | 2-Monopalmitin trimethylsilyl ether | C25H54O4Si2 | ![Structure](image7) |
| 33 | 19.22          | 1.47       | D-Galactose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)- | C21H52O6Si5 | ![Structure](image8) |

(continued on next page)
safe to be use in experimental animals (Ganaie et al., 2015; Subramaniyan et al., 2018). However, the effect of \textit{R. vesicarius} on embryonic development has never been tested before in pregnant animals. The zebrafish screening assays done in this study has revealed that the crude extracts prepared from various parts of \textit{R. vesicarius} did not induce teratogenicity or toxicity in developing zebrafish embryos even at very high concentration. The effect of \textit{R. vesicarius} on fetus development need to be tested in mammalian animal models but the zebrafish developmental toxicity screening results from this study suggest, it could be a safer choice of antiangiogenic medication in pregnant cancer patients as well.

6. Conclusion

The crude methanol extract of stem of \textit{R. vesicarius} has shown significant level of antiangiogenic activity, while being nontoxic

| Table 3 (continued) |
|----------------------|
| No | Retention time | PECENTAGE | Compound name | Formula | structure |
| 34 | 23.974 | 4.69 | Hexadecanoic acid, ethyl ester | C18H36O2 |
| 35 | 29.919 | 2.52 | 9,12-Octadecadienoic acid, ethyl ester | C20H36O2 |

| Table 4 |
|-----------------------------|
| Identified compound | Protein targets | Common name | Uniport ID | Role in angiogenesis | Reference |
| Propanoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester | Leucine-rich repeat serine/threonine protein kinase 2 | LRRK2 | Q5S007 | LRRK2 inhibitor (BAY 43–9006) proved substantial activity against (VEGFR)-2, VEGFR-3 modulation of angiogenesis by all Adenosine receptors | (Wilhelm et al., 2004) |
| Adenosine A receptors | | ADORA1 | P30542 | | |
| | | ADORA2A | P29274 | | |
| | | ADORA2B | P29275 | | |
| FK506-binding protein 1A | | FKBP1A | P62942 | antiangiogenic activity by FKBP1 and its peptide “expressed by endothelial cells and are target for therapeutic modulation of angiogenesis” | Yakkundi et al., 2015 |
| Muscarinic acetylcholine receptor M4 | | CHRM4 | P08173 | | (Clark et al., 2007) |
| Vascular endothelial growth factor receptor 2 | | KDR | P35968 | Endothelial cell migration and proliferation. | Miettinen et al., 2012 |
| Butanedioic acid, bis (trimethylsilyl) ester | c-Jun N-terminal kinase | MAPK8, MAPK10, MAPK9 | P45983, P45984 | activation of MAPK signaling by VEGF | Song and Finley, 2018 |
| | Phosphodiesterase 5A | PDE5A | O76074 | PDE5A inhibitors stimulates angiogenesis | Zhu et al., 2009 |
| | Serine/threonine protein phosphatase PPI-alpha catalytic subunit | PPP1CA | P62136 | Reverse Genetic Screen identify PPP1CA as Novel Angiogenesis Targets | Kalen et al., 2009 |
| Butane, 1,2,3-tris (trimethylsiloxy) | Presenilin 1 | PSEN1 | P49768 | Endothelial progenitor cells growth and differentiation | Boulton et al., 2008 |
| | Presenilin 2 | PSEN2 | P49810 | | Miettinen et al., 2012 |
| | Vascular endothelial growth factor receptor 2 | KDR | P35968 | Regulates endothelial migration and proliferation. | Chen et al., 2019 |
| | Fibroblast growth factor receptor 1 | FGFR1 | P11362 | Roles in metastasis and angiogenesis of breast cancer. | |
to zebrafish embryos. Novel bioactive molecules, which target antiangiogenic regulating proteins have been identified in the stem of R. vesicularis. The chloroform extract of stem was also cytotoxic to human breast, colon and liver carcinoma cells lines. 

Based on the results from this study, it is recommended that formulation prepared from Rumex vesicularis L could further be tested in clinical trials to evaluate its therapeutic potential as an effective and safe anticancer agent and remedy to treat diabetic retinopathy.

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Declaration of Competing Interest

No conflict of interest to be declared.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sbsj.2019.11.042.

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