Evaluation of crude saponins, methanolic extract and subsequent fractions from *Isodon rugosus* Wall. ex Benth: Potentials of anti-angiogenesis in egg and anti-tumorigenesis in potato

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Abstract: Based on the ethnomedicinal use of *Isodon rugosus* the current study was designed to evaluate its crude saponins (Ir.Sp), and subsequent fractions for anti-angiogenic and anti-tumor potentials. Chorioallantoic membrane (CAM) assay was used in anti-angiogenic potentials with Dexamethasone as positive control. The antitumor activity was evaluated with potato disk method using Vincristine sulfate as positive control. Moreover, antibacterial activity was also conducted against *A. tumefaciens.* The highest anti-angiogenic effect was observed with Ir.Sp, i.e., 79.00±0.58% at concentration of 1000 µg/ml which drop drown to 48.67±1.20% at lowest tested concentration of 31.25 µg/ml with IC_{50} of 41 µg/ml. Similarly, in the anti-tumor activity the Ir. Chf revealed excellent inhibition of tumor with IC_{50} value of 60 µg/ml. All the samples (excluding Ir. Sp and Ir. Cr) were inactive against *A. tumefaciens*, which demonstrates that the samples which did not show any antibacterial activity are rich in certain bioactive principles which may be responsible for the anti-tumor and anti-angiogenic potentials. Our results conclude that the Ir.Sp, Ir.Chfmay be good targets for isolation of bioactive compounds responsible for the inhibition of excessive proliferation of cells and angiogenesis.

Keywords: *Isodon rugosus*, Potato disc, *Agrobacterium tumefaciens*, anti-angiogenic, antitumor, CAM.

INTRODUCTION

Angiogenesis or neo-vascularisation is an advanced and complicated process involving the activation, adhesion, proliferation and rebirth of epithelial tissue cells from pre-existing blood vessels. It plays a vital role in traditional physiological methods like healing of wound and regeneration. However, in addition excessive angiogenesis may also be the part of variety of pathological processes, for example inflammatory diseases, diabetic retinopathy and also the growth of solid tumor (Ayaz *et al*., 2016). In the malignancy of tumors and other angiogenesis dependant diseases various reports have demonstrated to the vital role of neo-vascularization. Anti-angiogenic medical aids that targets activated epithelium cells possess sound benefits over medical aid directed against tumor cells. Anti-angiogenic approach of cancer therapy represents a promising new strategy to cancer treatment, which will not be susceptible to drug resistance (Boehm *et al*., 1997). Since tumors depend upon angiogenesis for growth and metastasis, so inhibition of this process could indirectly inhibit tumor cell growth and cause tumor dormancy. Several anti-angiogenic agents have been developed for targeting angiogenic factors or their receptors, extra cellular matrix compounds and tube-shaped structure of epithelium (Kim *et al*., 2000).

Several steps are involved in metastasis or spreading of the cancer in the body. Initially the surrounding tissues and blood or lymphatic vessels are invaded by the cancerous cells sanctioning them to approach distant organs to form metastatic colonies. Thus interruption in the invasion of cancerous cells is a vital target in cancer therapy, as this is an important step in metastasis that could be a major cause of death in patients suffering from cancer (Thompson and Price 2002).

The development of novel anticancer agents from plant sources including vincristine and vinblastine offer convincing evidence that plant is potential source of novel anticancer drugs (Nia *et al*., 2004). The isolation of bioactive compounds from herbs have become the focus of numerous investigators due to their better efficacy and biodegradable nature. Several secondary metabolites including alkaloids, glycosides, saponins, flavonoids, terpenoids have been reported for cytotoxic, antitumor, anthelmintic, insecticidal and a variety of pharmacological properties (Rice-Evans *et al*., 1997; Ikeda *et al*., 2000; Francis *et al*., 2002; Kamal *et al*., 2017; Lage *et al*., 2010). Being the source of numerous useful compounds and diverse pharmacological activities, the plants have attracted the attention of large number of researchers around the world.

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Isodon rugosus belongs to *Lamiaceae*, a family rich in species containing large number of pharmacologically active principles. Several species of *Isodon* are used as anticancer, antimicrobial, antioxidants and insecticidal agents in folk medicine (Collins and Charles 1987; Wojdyło *et al*., 2007). *I. rugosus* is used ethnomedicinally in the management of hypertension, tooth-ache, rheumatism, pyrexia, skin diseases, ear, nose, throat infections and in the treatment of intestinal disorders (Khan and Khatoon 2007; Adnan *et al*., 2012). This plant has also been reported to possess various pharmacological activities. Various species of the same genus have recently been reported to possess strong anticancer potentials with the isolation of bioactive principles i.e., Excisanin A from *Isodon macrocalyxin*, oridonin, ponicidin, xindongnin A, and xindongnin B from *Isodon rubescens* (Leung *et al*., 2005). In the present study *I. rugosus* was investigated for its possible anti-tumor and anti-angiogenic potential in an effort to explore novel sources of bioactive principles which can potentially be used in the treatment of angiogenesis dependent tumor and other neoplasia.

MATERIALS AND METHODS

Plant collection

The extraction process was carried out by taking the aerial part (stem and leaves) of *I. rugosus.* The plant was collected from the Safari valley district Dir, Khyber Pukhtunkhwa, and was identified by Dr. Ali Hazrat, Department of Botany, SBBU, Dir upper, Sheringal campus. The plant sample was deposited at the herbarium of the same university under voucher number (1016AZ). The plant material was washed with tap water for removing the soil particles and was converted into pieces. The plant was shade dried for 25 days and was converted into coarse powder with the help of grinder (Ahmad *et al*., 2015a).

Extraction

The coarse powder of the plant weighing 9kg was soaked in 20 liters of 80% methanol for 20 days. The filtration process was done and the filtrate was obtained by using rotary evaporator at temperature of 45°C. The concentrated residue obtained in round bottom flask was converted into semi-solid form by keeping in a water bath weighing about 550 grams. The crude methanolic extract obtained was subjected to fractionation through solvent-solvent extraction process. The Ir.Hex, Ir.Chf, Ir.EtAc and Ir.Aq obtained were 60, 170, 65 and 130 grams respectively (Shah *et al*., 2015).

Extraction of crude saponins

Plant powder (20gm) was taken in a conical flask and 100 ml of 20% ethanol was poured into it and shook. The plant sample was transferred to water bath and heated at 55°C for 4 h. The sample obtained was filtered and the concentrated sample was taken in a conical flask by adding 200 ml of 20% ethanol. The volume of sample was reduced to 40 ml by heating in water bath. Similarly the residue was transferred into the separating funnel and added 20 ml of diethyl ether. After vigorous shaking in conical flask two layers were obtained. The upper diethyl ether layer was discarded while the lower aqueous layer was collected. Then 60 ml of *n*-butanol was added into the aqueous portion and the funnel was shacken. The combined aqueous and *n*-butanol suspension in the funnel was washed with 10ml of 5% sodium chloride solution. The dried saponins were obtained by shifting the remaining solution into hot water bath by evaporating the solvent and 1.5gm of dried saponins were obtained.

Anti-angiogenic activity

In this study the fertilized chicken eggs were used to carry out chorioallantoic membrane assay (Ahmad *et al*., 2016; Farooq *et al.,* 2019). Eggs were placed in an incubator at 37^{o}C having the wide end up and were moved once in a day. After 4 days of incubation, the eggs were observed with the help of torch and made the circled the embryo-head. With the help of needle Albumin (1 ml) was aspirated from eggs through a small hole made at the upper slim end of the eggs, permitting the little CAM and yolk sac to urge away from the shell membrane. The covering of the shell above the air sac was removed aseptically using a forceps to expose the CAM surface for application of test samples. The cover-slip loaded with a sample was applied to the CAM surface. The chick embryo was then re-shifted to the incubator. After three days the methanol and acetone mixture (1:1) was injected into embryo chorioallantois using a needle. The CAM was separated from eggs and the number of vessels was observed and counted under a microscope. For each sample dose at least 20 eggs were used. The percent inhibition was calculated using the following equation:

$$\% \text{ inhibition} = (CAMns - CAMts / CAMns) \times 100$$

CAMns (number of blood vessels in CAM treat with normal saline)
CAMts (number of blood vessels in CAM treated with test samples)

Anti-tumor activity

A. tumefaciens-extract mixture preparation

For antitumor assay, stock solutions of plant extract were prepared and were serially diluted to concentration of 1000, 500, 250 and 125µg/ml and then filtered through sterile Millipore filter (0.22µm), into a 2ml sterile Ependorf tube. *A. tumefaciens* broth culture (0.1ml) were transferred to each of the previous sample tubes, containing sample concentration and then mixed well by gentle vortexing. Same procedure was followed for positive control i.e., vincristine sulfate while the distilled water having 0.1ml bacterial culture was used for negative control.

Preparation of potato discs

The surface potato tubers of moderate size were sterelized by immersing in 0.5% sodium dichloroisocyannurate solution for 30 min, washed in sterilized distilled water

and dried aseptically for 20min. The potato tuber was cut using a sterilized sharp cutter into small size discs having diameter of 1.5cm and thickness of 0.5cm. The discs were transferred into sterilized Petri dish having semi solidified agar media. The discs in petri dishes have to be inoculated with 50µl the plant extract-bacterium mixture. Same process was applied for positive control while for the distilled water which was used as negative control having bacterial culture was applied on potato disc. In this study each sample was used in triplicate. The Petri dishes were kept at 25°C for 20 days. After 20 days, staining the surface of the discs with iodine solution and the tumors on the discs were counted with the help of binocular (Amara, El-Masry *et al*., 2008).

Antibacterial assay

Preparation and Standardization of inoculums

Overnight grown culture of *A. tumefaciens* was used to prepare bacterial suspension and was adjusted to cell density of 1×10^6 CFU/ml using McFarland standard. Standardized the Suspension using UV-visible spectrophotometer and the standardization was maintained at 625nm during the entire trial.

Well diffusion assay

Antibacterial activity of *I. rugosuss* was investigated using previously reported procedure (Imran, Ullah *et al*., 2014; Kamal, Ullah *et al*., 2015). Nutrient agar media was inoculated with the test organism under laminar flow hood. Sterile cork borer was used to make wells (5mm) in each petri plate. Plant samples were serially diluted to prepared concentrations (10, 5, 2.5, 1.25mg/ml). Test samples (100 µl) were aseptically transferred to each well and petri plates were incubated at 37^{o}Cfor 24h. Cefotaxime was used as positive control. Correspondingly measured the zone of inhibition of all the test samples and the data obtained in triplicate was expressed as mean ± SEM.

Determination of MICs

MIC of various test samples was evaluated by broth dilution method. Stock solution was prepared for each sample having concentration of 5mg/ml. Various dilutions were prepared from stock solution using nutrient broth i.e., from 5mg/ml to 0.61µg/ml. Each sample was inoculated by adding bacterial suspension of 0.2ml to each sample using a sterile micropipette. These samples were incubated at 37^{o}C for 24h. The turbidity of each test sample was measured and the minimum inhibitory concentration at which there was no bacterial growth was considered as the MIC of a particular test sample (Imran, Ullah *et al*., 2014).

STATISTICAL ANALYSIS

Each trial was performed in three replicates and values were expressed as mean ± SEM. Two-way ANOVA followed by Bonferroni test was used for the comparison of positive control with the test groups. The *P* values less than or equal to 0.05 were considered as statistically significant. IC_{50} values were calculated by a linear regression analysis among the percent inhibition against the extract concentrations via Excel program. Cluster analysis was performed in SPSS version 16.0 following Ward's method to draw dendrogram hierarchical clusters.

RESULTS

Anti-angiogenic effect

The crude methanolic extact, various fractions and saponins isolated from *I. rugosus* revealed prominent anti-angiogenic activity in CAM assay as showed in table 1. The highest anti-angiogenic potential was presented by Ir.Sp in dose dependent manner i.e., 48.67±1.20, 52.33±0.33, 57.00±1.15, 63.67±0.67, 71.00±1.15, 79.00±0.58% at the concentrations of 31.25, 62.5, 125, 250, 500 and 1000 µg/ml with IC_{50} of 41µg/ml. Further, Ir.Chf also exhibited high inhibitory effect in CAM assay i.e., 44.00±0.58, 48.33 ±0.33, 52.67±0.88, 57.33±0.67, 67. 67±0.88, 74.33±0.33% at the concentrations of 31.25, 62.5, 125, 250, 500 and 1000 µg/ml with IC_{50} of 82µg/ml. From the analysis it is clear that all the test samples demonstrated a reasonable response in dose dependent manner. The effect shown by Ir.Sp was comparable with the positive control. The order of antiangiogenic activity for all the test samples was Ir.Sp>Ir.Chf>Ir.EtAc>Ir.Cr >Ir.Hex>Ir.Aq with IC_{50} values of 41, 82, 145, 375, 795, 1070 µg/ml respectively.

Antitumor effect

The antitumor assay carried out for various fractions of *I. rugosus* demonstrated a dose dependent antitumor response. Ir.Chf exhibited highest antitumor activity amongst all the test samples i.e., 86.67±1.93, 77.77±2.93, 70.00±3.83, 57.77±1.10, 50.00±1.93, 45.57±2.23% inhibition at concentration of 1000, 500, 250, 125, 62.5 and 31.25 µg/ml respectively with IC_{50} of 60 µg/ml. The Ir. Sp also revealed a significant antitumor potential i.e., 82.23±2.23, 70.00±1.93, 64.43±2.93, 53.33±3.83, 51.10±1.10, 42.23±2.23% at the concentration of 1000, 500, 250, 125, 62.5 and 31.25 µg/ml respectively with IC_{50} value of 65µg/ml. Similarly at 1000 µg/ml the Ir. Cr, Ir. Hex, Ir. Chf, Ir. Et Ac and Ir. Aq exhibited 76.67 ±1.93, 64.43±2.93, 86.67±1.93, 80.00±1.93 and 56.67±1.93% tumor inhibition with IC_{50} value 118, 200, 60, 68 and 460 µg/ml respectively.

Antibacterial effect

Antibacterial activity of the concerned test samples performed against *A. tumefaciens* established negligible action. This investigation was established to ensure that whether the test samples were active against this specific strain or not. The antibacterial assay summarized in the Table 3 showed that among the test samples, the Ir. Sp and

Table 1: Results of angiolytic assay of *Isodonrugosus* extracts and saponins

Samples	Conc. 31.25 µg/ml	Conc. 62.5 µg/ml	Conc. 125 µg/ml	Conc. 250 µg/ml	Conc. 500 µg/ml	Conc. 1000 µg/ml	IC_{50} µg/ml
Ir.Cr	31.00 ±0.58***	34.67 ±0.88***	39.33 ±0.88***	43.00 ±0.58***	56.33 ±0.67***	68.67 ±0.88***	375
Ir.Hex	28.33 ±0.88***	30.00 ±1.53***	36.00 ±1.15***	39.00 ±0.58***	43.67 ±0.67***	55.00 ±1.15***	795
Ir.Chf	44.00 ±0.58***	48.33 ±0.33***	52.67 ±0.88***	57.33 ±0.67*	67.67 ±0.88***	74.33 ±0.33***	82
Ir.EtAc	39.00 ±1.15***	43.00 ±1.15***	48.67 ±0.88***	56.00 ±0.58**	63.67 ±0.88***	70.33 ±0.33***	145
Ir.Aq	18.33 ±0.88***	23.67 ±1.45***	29.00 ±0.58***	31.33 ±0.33***	35.67 ±0.88***	44.00 ±0.58***	1070
Ir.Sp	48.67 ±1.20***	52.33 ±0.33***	57.00 ±1.15***	63.67 ±0.67ns	71.00 ±1.15***	79.00 ±0.58ns	41

Table 2: Results of antitumor of *Isodonrugosus*extracts and saponins

Samples	Concentrations (µg/ml)	Average inhibition	Percent inhibition	IC_{50} (µg/ml)
Ir.Cr	1000 500 250 125 62.5 31.25	23.00±0.58 18.33±0.33 16.00±1.15 15.33±0.88 9.00±0.58 8.67±0.33	76.67±1.93*** 61.10±1.10*** 53.33±3.83*** 51.10±2.93*** 30.00±1.93*** 28.90±1.10***	118
Ir.Hex	1000 500 250 125 62.5 31.25	19.33±0.88 16.00±1.15 15.67±0.67 13.00±1.15 8.00±0.58 7.33±0.33	64.43±2.93*** 53.33±3.83*** 52.23±2.23*** 43.33±3.83*** 26.67±1.93*** 24.43±1.10***	200
Ir.Chf	1000 500 250 125 62.5 31.25	26.00±0.58 23.33±0.88 21.00±1.15 17.33±0.33 15.00±0.58 13.67±0.67	86.67±1.93ns 77.77±2.93ns 70.00±3.83** 57.77±1.10*** 50.00±1.93*** 45.57±2.23***	60
Ir.EtAc	1000 500 250 125 62.5 31.25	24.00±0.58 22.33±0.33 18.00±1.15 17.67±0.33 14.33±0.88 11.00±0.58	80.00±1.93* 74.43±1.10** 60.00±3.83*** 58.90±1.10*** 47.77±2.93*** 36.67±1.93***	68
Ir.Aq	1000 500 250 125 62.5 31.25	17.00±0.58 15.33±0.33 12.67±0.67 10.00±1.15 8.33±0.88 5.00±1.15	56.67±1.93*** 51.10±1.10*** 42.23±2.23*** 33.33±3.83*** 27.77±2.93*** 16.67±3.83***	460
Ir.Sp	1000 500 250 125 62.5 31.25	24.67±0.67 21.00±0.58 19.33±0.88 16.00±1.15 15.33±0.33 12.67±0.67	82.23±2.23ns 70.00±1.93*** 64.43±2.93*** 53.33±3.83*** 51.10±1.10*** 42.23±2.23***	65

Table 3: Antibacterial (*Agrobacterium tumefaciens*) potential of various samples of *Isodonrugosus*

Samples	Concentrations (mg/ml)	Zones of inhibition	MIC (µg/ml)
Ir.Cr	10 5 2.5 1.25	13.00 ± 0.58 09.00 ± 1.15 Na Na	2630.00 ± 5.78
Ir.Hex	10 5 2.5 1.25	Na Na Na Na	----------
Ir.Chf	10 5 2.5 1.25	Na Na Na Na	----------
Ir.EtAc	10 5 2.5 1.25	Na Na Na Na	----------
Ir.Aq	10 5 2.5 1.25	Na Na Na Na	----------
Ir.Sp	10 5 2.5 1.25	14.67 ± 0.67 09.33 ± 0.88 07.00 ± 0.58 Na	1831.667 ± 4.41
Cefotaxime	10 5 2.5 1.25	31.33 ± 0.88 26.67 ± 0.67 22.33 ± 0.33 18.00 ±0.58	16.33 ± 0.88

Dexamethasone was used as positive control having IC_{50} value of 11.68 µg/ml, data is expressed as mean ± SEM (n= 3),* P<0.05, ** P<0.01, *** P<0.001. Key: Ir.Cr: Crude methanolic extract; Ir. Hex: *n*-hexane fraction; Ir. Cf: Chloroform fraction; Ir. EtAc: Ethyl acetate fraction; Ir.Aq: Aqueous fraction; Ir. Sp: Saponins.

Ir. Cr were active against the *A. tumefaciens*. There were no specific potential against *A. tumefaciens* among the rest of the samples. Similarly the result of Ir. Sp (MIC=1831.667±4.41 µg/ml) and Ir.Cr (MIC=2630.00 ±5.78 µg/ml) were insignificant in comparison with the positive control (MIC for cefotaxime =16.33±0.88 µg/ml) which reveals that the *I. rugosus* is not active against *A. tumefaciens*. In short, the antibacterial assay of *I. rugosus*

against *A. tumefaciens* does not disclose any considerable antibacterial potential in comparison with positive control.

DISCUSSION

Medicinal plants are used worldwide for the prevention and treatment of various diseases (Ali *et al.,* 2017; Ahmad *et al*., 2015a; Ahmad *et al*., 2015b; Ahmad *et al*., 2016). Majority of world's population depends upon natural sources, in which the herbs are mainly used to cure and treat different diseases in complementary and alternative medicine (Abbasi *et al*., 2010; Ullah *et al.,* 2016). Attention has been focused upon the isolation of bioactive compounds from medicinal plants which may lead to novel drug development to get remedies for various challenging diseases like neoplasia and infectious diseases. In neoplasia, excessive proliferation of cell occurs with excessive angiogenesis in the affected sites while in normal human body the process of angiogenesis is carried out in a sternly controlled manner which is synchronized by various angiostatic factors (Wang *et al*., 2004). Many physiological processes such as normal reproductive function, wound healing and embryonic development come under normal angiogenesis process (Zetter 1998). However, abnormal angiogenesis is a decisive factor in the pathogenesis of many more diseases including cancer, rheumatoid arthritis, diabetic retinopathy (Lee *et al*., 1998; Carmeliet and Jain 2000; Bodolay *et al*., 2002). Every step of these angiogenesis processes frequently act as a potential target for the therapeutic interference of the angiogenesis. Tumor growth and metastatic action can be prevented by Inhibition of angiogenesis process (Choi *et al*., 2007). Various plants that contains a diverse bioactive compounds with angiogenesis-modulating potential have been previously reported (Fan *et al*., 2006). In the similar way, our current investigational study also conforms to the previous reports of plants regarding the CAM assay and potato tumor assay. The chick chorioallantoic membrane assay was used for evaluation the anti-angiogenic activity of *I. rugosus* which may is a sound indication of antitumor potential. Similarly in the potato tumor assay the similar results have been observed for various samples. Potato tumor is a neoplastic disease characterized by crown gall caused by *A. tumefaciens* (Gram-negative bacterium) (Galsky *et al*., 1980). The symptoms of infection caused by *A. tumefaciens* are similar to tumor in mammalian cells. The Ti-plasmid of bacterial cell infect the plant's cells resulting in tumor formation similar in nucleic acid content and histology to human and animal tumor (Agrios 1997). Crown gall tumor can be inhibited on potato discs with sound correlation with samples active against 3PS (murine leukemia) (Galsky *et al*., 1980). The validity of potato disc bioassay is anticipated on the observation that certain tumor induction mechanisms are similar in plants and animals like fast multiplying without apoptosis (Braun 1971; Becker 1975; Karpas 1982). Our current investigations compiles a good profile of anti-tumor and anti-angiogenic potentials of *I. rugosus*, however, further study is required to demonstrate the necessary active component in various fractions of this plant especially Ir. Sp and Ir. Chf and to explore valuable role of *I. rugosus*as a possible remedy for neoplasia.

CONCLUSIONS

The CAM assay and anti-tumor assay of various samples of *I. rugosus* showed remarkable results. It has also been observed that all the samples (excluding Ir. Sp and Ir. Cr) of *I. rugosus* were inactive against *A. tumefaciens*, which demonstrates that the samples which didn't show any antibacterial activity are rich in certain bioactive principles which may be responsible for the anti-tumor and anti-angiogenic potentials. Based on the results of our current investigational study it may be inferred that *I. rugosus* possess strong anti-tumor and anti-angiogenic potentials. The results also demonstrate that the Ir. Sp and Ir. Chf of *I. rugosus* may be good sources of bioactive principles responsible for the inhibition of excessive proliferation of cells and angiogenesis.

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