Journal of Animal & Plant Sciences, 32(3): 2022, Page: 736-745
ISSN (print): 1018-7081; ISSN (online): 2309-8694
http://doi.org/10.36899/JAPS.2022.3.0475

MICROMORPHOLOGY, PHYTOCHEMICAL AND PHARMACOLOGICAL EVALUATION OF *Isodon rugosus* (Wall. ex Benth.) Codd.

A. Hameed[1], M. Zafar[1], M. Ahmad[1], S. Sultana[1], M. S. Akhter[2], W. Zaman[3], S. Saqib[4,5] and F. Ullah[5,6*]

[1]Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan
[2]Department of Chemistry, College of Science, University of Bahrain, Bahrain
[3]Department of Life Sciences, Yeungnam University, Gyeongsan 38541, Gyeongbuk, Republic of Korea
[4]State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China
[5]University of Chinese Academy of Sciences, Beijing 100049, China
[6]CAS Key Laboratory of Mountain Ecological Restoration and Bioresource Utilization, Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, China
Corresponding author's Email: fazalbotanist@gmail.com

ABSTRACT

Isodon rugosus Wall. ex Benth (IR) is one of the ethnomedicinal important plants of Gilgit-Baltistan, Northern Pakistan. The present study aims to evaluate the micro-morphological features, phytochemical screening, and pharmacological potential of IR. SEM and LM were used as an identification tool. Five different solvents were used to prepare IR extracts. Phytochemical and antioxidant activities were determined calorimetrically. To investigate antidiabetic, α-amylase inhibition assay was adopted. Cytotoxicity was tested using a brine shrimp assay. Anti-leishmanial via MTT assay. Disc-diffusion assay was used for protein kinase inhibitory, antibacterial, and antifungal activities. Pollen was of monad, hexacolporate, and circular shape. Lophate sculpturing with outer exine pattern elevated on the rides. Seeds were oblong-ovate, small projected with smooth-rough, variously ridges/wrinkled sculpturing. Epidermis cells were irregular in shape with a slightly straight anticlinal wall. Stomata with diacytic, and unicellular glandular trichome were observed. The extracts were rich in phytochemicals, the maximum amount of phenolic and flavonoid contents was found in methanol extract (IRM) 89.76mg GAE/gm and 85.69mg QE/gm. All the extracts show substantial antioxidant activity but highest in IRM (DPPH IC_{50} 44.51 µg/ml, total antioxidant capacity 93.60 mg AAE/g, and total reduction power of 93.44 mg AAE/g). Potential antibacterial and antifungal activities were reported for IRM. Significant protein kinase, alpha-amylase inhibition, and cytotoxic activity were revealed. Dose-dependent cytotoxic activity was exposed against *Leishmania tropica* (LC_{50} 11.16 µg/mL). In conclusion, *I. rugosus* extracts have shown potential biological applications and should be subjected to further research work to develop further biomedical applications.

Keywords: *Isodon rugosus,* SEM, LM, phytochemicals, antimicrobial, cytotoxicity, alpha-amylase inhibition.

Published first online October 20. 2021 Published final May 30. 2022

INTRODUCTION

Plants are serving mankind from the beginning of their creation and are still being used as the major constituent of herbal medicines. World Health Organization, documented that more than eighty percent population, using plant-based drugs. Therefore, scientists of the present day are paying considerable attention to natural resources amongst them the plants are considered best (Khan *et al*., 2019). Family Lamiaceae consists of 236 genera and 7200 species. The Mediterranean region and Central Asia have a diverse variety of Lamiaceae (Perveen and Qaiser, 2004). *Isodon* with 100 species in the genus of the family Lamiaceae. It comprises perennial herbs, subshrubs, and shrubs. Plants from this genus are the richest in diterpenoids and greater than 300 are being extracted (Neelamkavil and Thoppil, 2017). *Isodon rugosus* Wall. ex Benth of family Lamiaceae, are locally recognized as Sperkai, Boi, and Phaypush. The aromatic plant, 1-5ft tall, branched shrub that has an erect stem, ovate shape leaves with notched margin, and small dense hairs on the ventral side. The flowering period ranges from July to September and the seedling period ranges from August to October (Sadiq *et al*., 2018a). Traditionally this plant is utilized for the treatment of toothache, bronchodilator, diarrhea, and antiseptic. Extract from fresh leaves are used to cure scabies, earache, fevers, hypertension, toothache, and rheumatism. Different extracts and fractions of *I. rugosus* exhibited antibacterial, antifungal, and antioxidant activities (Janbaz *et al*., 2014). Previous studies on phytochemicals revealed the occurrence of flavonoids, saponin, cardiac glycosides, reducing sugars, terpenoids, coumarins, and steroids (Zeb *et al*., 2014).

Adulteration in broader terms is the replacement of one substance with another, that has poor composition

as compared to the original one (Hameed *et al*., 2020). According to Mitra and Kannan (2007), sometimes adulteration may occur in the transportation of raw materials. This may occur due to shortage of medicinal plants and an increase in its demand leading to adulteration of the original crude drug. LM and SEM microscopic techniques were applied for species identification, characterization, and pharmacogenetics parameters in recent years by many researchers (Gul *et al*., 2019; Sufyan *et al*., 2018). The knowledge provided by this research work will contemplate the therapeutic potential of *I. rugosus*, as well as the implementation of LM and SEM for its correct identification in order to lessen the adulteration.

MATERIALS AND METHODS

Plant collection and Preparation of crude extracts: Collection of *I. rugosus* was carried from the Northern regions of Pakistan (Gilgit-Baltistan). Fresh plant specimens during August 2019 along with 22 inflorescences were collected from studied plants located at Nagar, Gilgit-Baltistan, Pakistan (36° 16' 36" N, 74° 43' 10" E). Seeds were also collected (September 2019). Arial parts of *I. rugosus* were washed and at room temperature, the plants were dried. The dried plant samples were ground to fine powder (1mm diameter). The grinded plant powder (25g) was soaked in 0.5 L of methanol, ethanol, chloroform, ethyl acetate, and n-hexane for 15 days at room temperature. Extracts were further label as IRM, IRE, IRC, IEA, and IRH for methanol, ethanol, chloroform, ethyl-acetate, and n-hexane extracts of *I. rugosus*.

LM and SEM of Pollen and seeds surface morphology: Mature pollen from ripens anthers was separated and mounted onto the glass slide. The pollen was crush using few drops of acetic acid. Debris was removed with the help of a needle. Glycerin jelly was used for staining purposes and the glass slide was fixed with a cover slip. Different characters such as colpi width, colpi length, polar diameter, exine thickness, and equatorial diameter were examined were examined for pollens. Photographs were taken by using an infinity camera of LM (Naz *et al*., 2019). Micro-morphological characters such as seed coat and surface were studied with the help of SEM (Model JEOL JSM- 5910) (Munir *et al*., 2019; Ayaz *et al*., 2020).

Foliar epidermal anatomy using LM: Leaves epidermal anatomy was done by the adopted methodology of Sadia *et al* (2019), but with little modification. Fully dried samples of leaves were utilized for analysis. Few parts of the leaf were firstly boiled in a mixture of nitric acid and lactic acid (1:3) until the leaves showed transparent color. Quantitative and qualitative characters such as stomatal pore size, stomata size, epidermal cell shape and size, trichomes number were measured with the help of a light microscope.

Total phenolic content (TFC) and total flavonoid contents (TFC) determination: Folin-Ciocalteu modified method was used for the determination of TFC (Wen *et al*., 2015). Gallic acid as a positive controller and pure DMSO as a negative controller were used.

TFC of plant sample was calculated with the help of spectrophotometer UV method (Chang *et al*., 2002). Quercetin and pure DMSO as positive and negative stander were utilized. Absorbance at 405 nm was taken using a microplate reader.

Antioxidant Assays

DPPH radical scavenging antioxidant activity: The antioxidant capacity of extracts was measured by using the modified method of Phull *et al* (2016). As a standard ascorbic acid was used. Further, calculations were made for scavenging activity by using the given equation.

%age Scavenging Capacity = (1 – optical density of plant extract) / (optical density of positive control) ×100

Phosphomolybdenum assay: The modified method of Phull *et al* (2016) was used for the determination of the Phosphomolybdenum antioxidant capacity of given plant extract. Ascorbic acid is used as standard.

Total reducing power detection: Plant extract (100 µl) was added with 0.2M of phosphate buffer (200 µl) into the solution of 10% trichloroacetic acid (240 µl) in the Eppendorf tube. The whole reaction mixture was incubated for 10 minutes and after this at 3000 rpm centrifuging the mixture. The supernatant (150 µl) mixed with ferric chloride (50 µl) solution. On microplate reader at 630 nm readings were taken, Ascorbic acid as a standard was used (Chaves *et al*., 2020).

Antimicrobial Activity

Antibacterial and antifungal activity: Method of disc-diffusion was used for measuring both antibacterial and antifungal activity. *S. aureus* ATCC 6538, *B. subtilus* ATCC 19659 gram-positive strains and *P. aeruginosa* ATCC 90271, *K. pneumonia* ATCC 1705, *E. coli* ATCC 33456 gram-negative bacterial strains were used. Different fungal strains such as *M. racemosus* FCBP 0300, *C. albicans* FCBP 478, *A. niger* FCBP 0918, *F. solani* FCBP 0291, and *A. flavus* FCBP 0064 were used. Filter discs with plant extracts (200 µg) were transferred to media plates. After incubation of 48 hr at 28 °C, the zone of inhibition was measured. MIC at different concentration (100, 33.33, 11.11, 3.7 µg/ml) were measured.

Anti-leishmanial activity: The modified method of Khan *et al* (2014) was utilized for the *in-vitro* anti-leishmanial activity of *I. rugosus* extracts against

Leishmania tropica Kwh_{23} promastigotes strain. Amphotericin B and pure DMSO were used for +ive and -ive controls.

Cytotoxicity Assay

Brine shrimp lethality test (BSLT): A lethality test with some modifications was used for the assessment of cytotoxicity of *I. rugosus* extracts. *Artemia salina* eggs in the artificial seawater (3.8 g/L) under light incubated for 36–48 hr at 37°C. After harvesting the mature 10 phototropic nauplii were transferred into the microplate valves, having seawater and tested plant extracts. Doxorubicin and pure DMSO as a standard were used. After incubation of 24 hours in each valve, dead brine shrimps were counted with the help of LM (Apu *et al*., 2013). The values of LC_{50} were deliberate through the help of Table curve 2D version

Protein Kinase Inhibition (PKI) test: PKI potential of *I. rugosus* extracts was measured through the method as described by Yao *et al* (2011): Muhammad *et al*. (2014). Surfactin (20 µg) and pure DMSO as a positive and negative controller was used.

Alpha-amylase inhibition (AA) test: *In-vitro* AA inhibition test was performed on tested plant extracts through the previously described method Khalil *et al* (2018). Different doses of tested samples (250, 200, 100, 50, 25, 12.5, 6 µg/mL) were used. For standard Acarbose was utilized.

RESULTS AND DISCUSSION

I. rugosus widely distributed in Pakistan, Afghanistan, Oman, China, the Himalayas to Nepal. Ethno-medicinally *I. rugosus* used against wound healing, antibacterial infection, generalized body pain, abdominal pain, analgesic, earache, cytotoxicity, anticholinesterase, skin, gastric problems, angiogenic, anti-tumor and toothache. Alkaloids, saponins, flavonoids, tannins, phenolics, terpenoids, glycosides, anthraquinones, and triterpenes were the phytochemicals that were previously reported in this medicinally important plant (Sadiq *et al*., 2019; Khan *et al*., 2019; Zeb *et al*., 2017; Table 1).

Table 1: Botanical names, Voucher no, Family, Collection site, Growth form, Ethno-medicinal uses, reported Phytochemicals and Distribution of Collected Plants.

Botanical name, Voucher no	Local name	Family	Collection Site	Ethno-medicinal uses	Reported Phytochemicals	Distribution in World
***Isodon rugosus* (Wall. ex Benth.) Codd.** AH 11	Sperkai, Boi and Phaypush	Lamiaceae	Gilgit (Hunza, Nagar)	Wound healing, generalized body pain, analgesic, antibacterial property, abdominal pain, cytotoxicity, earache, anticholinesterase, skin, gastric problems, angiogenic toothache, and anti-tumor (Malik *et al*., 2019), (Sadiq *et al*., 2018b), (Siddiquah *et al*., 2018), (Janbaz *et al*., 2014)	Flavonoids, alkaloids, phenolics, saponins terpenoids, tannins anthraquinones, glycosides, triterpenes, (Sadiq *et al*., 2019), (Khan, S. *et al*., 2019), (Zeb *et al*., 2017)	Pakistan, Afghanistan, Oman, China Himalayas to Nepal.

Taxonomic clarification: Plant taxonomic characterization are important for the proper identification of different plant species. Different researchers in many studies had introduced various techniques for the identification of original drugs but these all techniques are expensive. Therefore, researchers are paying attention to less expansive techniques, among them palynological and taxonomic techniques were the most simple, useful, and inexpensive (Rashid *et al*., 2019).

In the present study different quantitative and qualitative characters were studied including pollen, seed morphology, and leaf epidermis anatomy. Flowers of *I. rugosus* were whitish with dense purple veins projecting from outside. Seeds were blackish. Leaves of *I. rugosus* were an ovate shape, with a notched margin and small dense hairs were also present on the ventral side. Pollen of *I. rugosus* were monad and hexacolporate. The shape of pollen in polar view was circular and intersemi angular and in equatorial view sub-prolate. The polar diameter was 16.5±2.1 µm (14.3-18.6 µm) and the equatorial diameter was 16.6±1.7µm (14.6-19.3 µm). The polar/Equatorial ratio was 0.9. Colpi length was 5.6±1.5 µm (4.5-6.8µm) and width 2.7±1.5 µm (2.5-3.7 µm) Thickness of exine 1.6±1.2µm (1.4-1.8 µm). Sculpturing

was lophate, with outer exine pattern elevated on the rides as given in Plate1 (A-F).

Seeds micro-morphological characters along with seed size and seed shape, were the diagnostic outfits for the proper identification of different plant species (Luqman *et al*., 2019). Seed color was black, with length and width of 0.2–0.4 cm and 0.6–0.8 cm. When visualized by SEM the seeds of *I. rugosus* were seen to be oblong-ovate with small projection, cells were polygonal elongated, Sculpturing was smooth-rough, variously ridges or wrinkled with few projections, margins were wavy slightly straight or deeply dentate, the anticlinal wall was somewhat deep while the outer periclinal wall was flat to concave as given in Plate 1(G-L).

Implementation of foliar epidermal anatomy in the field of systematic botany is nowadays dominant just similar to the usage of other bio-markers e.g. chemical compositions and DNA sequence (Mbagwu and Edeoga, 2007). Both adaxial and abaxial surfaces of *I. rugosus* were studied to examine the leaf epidermis features. Epidermis cell of irregular shape with the slightly straight anticlinal wall. A significant difference in the size of the epidermis cells on both adaxial and abaxial surfaces was observed. At adaxial side the epidermis cell length and width were 15-25 = 20.5±3.03 µm, 11-17.8 = 14±1.7 µm and the abaxial side, length and width of epidermis cells as 12-23 = 18.3±2.4 µm, 9-16 = 12.6±1.22 µm. Subsidiary cells were found on both sides. In adaxial side, subsidiary cell with length 16-22 = 19.3±1.82 µm and width 7.51-13 = 10.4±1.45 µm whereas on abaxial side, length and width of the subsidiary cell was of 15-24 = 19.2±2.76 µm and 5.7-14 = 9.9±1.56 µm. Diacytic types of stomata were present on both sides. In adaxial side, stomatal length was 15-21.4 = 18.9±1.6 µm and stomata width 6.6-11 = 9.5±0.9 µm and on abaxial side stomata length and stomata width was 13-23 = 18.3±1.8µm and 18.76-22.60 = 20.26±0.62 µm. Guard cell, present on both adaxial and adaxial sides with length and width 17-23.6 = 20.5±1.8 µm, 7.51-8.26 = 7.9±0.37µm and in abaxial side with the length and width 12-17.6 = 15.6±0.96 µm and 5.6-10.5 = 8.3±1.06 µm on abaxial surface respectively. Trichome was unicellular glandular type and was present on only the abaxial surface of *I. rugosus* (Plate 1 M-O). Agreeing to Ahmad *et al* (2010) shapes and types of epidermis cells, trichomes, stomata, and glandular cells were the most important features in anatomical studies and usually serving as a support in the field of taxonomic verification.

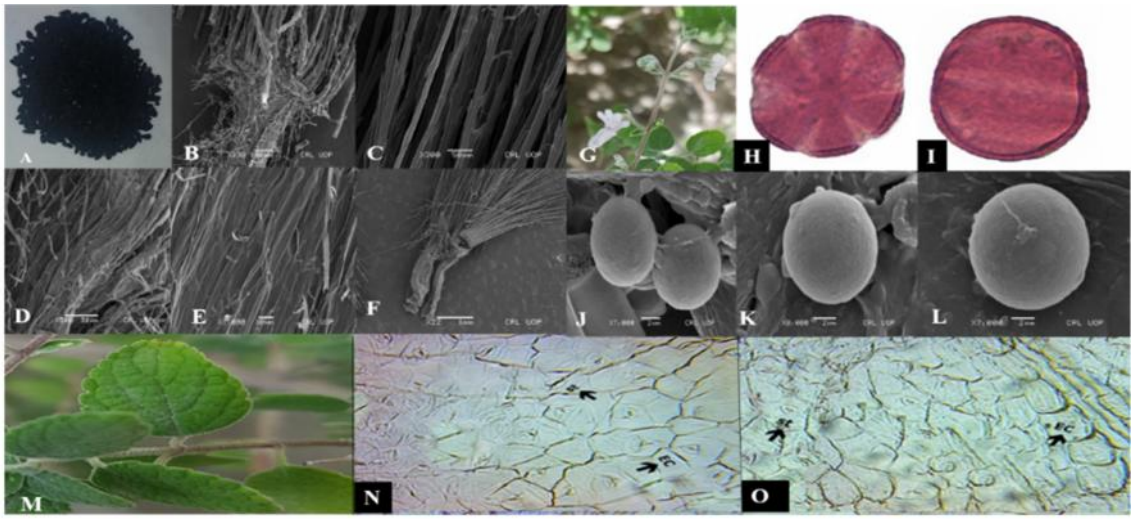

Plate 1: *I. rugosus*; field photograph of seeds photograph (A), SEM of seed micrographs (B-F). field photograph of Flower (G), LM of *I* Pollen micrographs (H-I), SEM Pollen micrographs (J-L). Field photograph of leaves (M), Foliar anatomical micrographs of showing epidermal cell (EC), stomata (st), trichome (tri) on adaxial and abaxial surfaces(N&O).

Quantitate phytochemical analysis of various extracts of *I. rugosus*: An inclusive range of biological properties are observed by phenolic compounds such as antiatherogenic, antibacterial, antithrombotic, antiallergenic, antioxidant, anti-inflammatory, cardioprotective, and vasodilatory effects. Previously reported that the phenolic compounds possess positive relation with antioxidants (Csepregi *et al*., 2020). The results of phenolic contents of *I. rugosus* various extracts are summarized in Figure 1. IRM has the highest phenolic content (89.76 mg GAE/gm), followed by IRE (80.89 mg GAE/gm), IRC (75.33 mg GAE/gm), IRH (51.14 mg GAE/gm), and IRA (44.40 mg GAE/gm).

Flavonoids belong to the class of polyphenolic compounds, that have an extensive range of pharmaceutical properties such as anti-inflammatory,

anti-allergic, anti-hepatotoxic, anticancer, and anti-viral properties. Naturally, flavonoids are produced in all plant parts and have positive applications on human health (Ruwali and Negi, 2019). IRM has the highest flavonoid content (85.69mg QE/gm), followed by IRE (73.96 mg QE/gm), IRC (63.37 mg QE/gm), (57.35 mg QE/gm), and IRA extract (46.50 mg QE/gm). Our findings were in agreement with Howlader *et al* (2016), mentioned that methanol is the best solvent for phenolic and flavonoid contents.

Antioxidant potential of various extracts of *I. rugosus* : The scavenging activity of *I. rugosus* extracts for radicals was calculated by DPPH assay. DPPH radical scavenging assay was a very short time consuming and sensitive assay for the determination of the antioxidant capacity of plant extracts (Martinez-Morales *et al*., 2020). IC_{50} values for IRM showed the best results (44.51 ± 2.2 µg/mL) followed by IRE (46.32 ± 2.5 µg/mL), IRC (62.38 ± 3.2 µg/mL), IRA (123.7 ± 2.5 µg/mL), and IRH (229.6 ± 3.5 µg/mL). The concentration-dependent activity was observed as shown in Figure 1. The presence of phenolic and flavonoid contents in plant extracts gives them scavenging capabilities. Mostly the phenolic and flavonoids were extracted in large quantities by using polar solvents. Our findings were in accordance with Nguyen *et al* (2020) depicting that there is a direct connection between the phenolic contents and antioxidant capacity.

The total antioxidant potential of plant extracts was mostly assessed by the phosphomolybdate test. The principal of this assay was an alteration of molybdenum (VI) to molybdenum (V) by plant extracts which have antioxidant potential and give results in green color (Gupta *et al*., 2016). The highest antioxidant capacity was given by IRM (93.60±2.34 mg AAE /g sample) followed by IRE (82.40±2.24 mg AAE /g sample), IRC (76.57±2.61 mg ascorbic acid/g sample), IRA (70.39±2.26 mg AAE /g sample), and IRH (68.61 ± 2.76 mg AAE /g sample). Our findings have been in agreement with the previous study of Talbi *et al* (2020) that methanolic plant extract exhibited antioxidant capacity. IRM showed the maximum reducing power with 93.44± 2.38 mg AAE/g sample, followed by IRE (82.43±2.13 mg AAE/g sample), IRC (71.65± 2.35 mg AAE /g sample), IRA (51.13± 2.21 mg AAE /g sample), and IRH (43.87± 2.22 mg AAE /g sample) as shown in Figure 1. It was noticed that there was a strong relationship between the reducing power and with TPC (Islam *et al*., 2013). The present study has been in the agreement of Sahreen *et al* (2014) that methanol plant extract was the utmost sample in the reducing power assay.

Antimicrobial activity: Qualitatively and quantitatively antibacterial activity of *I. rugosus* was evaluated in contradiction of five bacterial strains. The preliminary screening results of bacterial strains were given in Table 2. IRM has a strong anti-bacterial capacity besides all tested bacterial strains, The extremely sensitive bacterial strain substantiated to be *Staphylococcus aureus* (34.5mm), *Klebsiella pneumoniae* (33.33mm), and least inhibition against *K. pneumonia (*15.53 mm). IRC showed antibacterial activity against all tested bacterial strains excluding *Bacillus subtilis and K. pneumonia.* IRH extract tested in contradiction of all selected bacterial strains but it did not give acceptable ZOI. In our results, IRM contains the highest amount of flavonoids and phenolic contents, which was in good correlation with finding Mahboubi *et al* (2015) in which authors described methanol extract have pronounced antibacterial activity as compared to other solvents that are used for extraction.

The antifungal potential of all extracts of *I. rugosus* was assessed by using the disc diffusion method in contrast to five fungal strains. IRM, IRE, and IRC showed significant ZOI whereas IRA and IRH did not give any activity (Table 3). The most susceptible fungal strain towards IRM *Mucor racemosus* (26.5 mm) while the less susceptible strain was *Fusarium solani* (ZOI 18.2 mm). Present findings are in compliance with the findings of Karaman *et al*. (2003), that concludes that the *Juniperus oxycedrus* methanol extract has strong antimicrobial property as compared to other extracts.

Leishmaniasis is a disease that is transmitted with the help of a vector, with visceral leishmaniasis in 500,000 cases and cutaneous 1–1.5 million cases occurring annually (Maina *et al*., 2020). According to World Health Organization (WHO), in about 88 countries leishmaniasis is endemic including Pakistan, India, Sudan, and Afghanistan and about 350 million people are at risk. Due to a lack of inadequate motivation towards developing affordable drugs and unsatisfactory vector control both in treatment and prevention, there is a higher risk of the separation of leishmaniasis (Goncalves *et al*., 2020). Therefore, *I. rugosus* were evaluated contrary to the *Leishmania tropica* , and results are given in Figure 1. The noticeable inhibition in growth of *L. tropica* was displayed by IRA and IRH IC_{50} 27.62±1.32 and 11.16±1.12 µg/mL. The reasonable activity was revealed by IRC extract, whereas no activity was found in IRE and IRM (Figure 1). Our results were in exact correlation with Zahra *et al* (2017) who reported the anti-leishmanial activity of nonpolar solvents.

Cytotoxicity assessment: The cytotoxic potential of *I. rugosus* extracts was tested against brine shrimp larvae to explore the cytotoxic potential. Different extracts of *I. rugosus* were initially screen for cytotoxic potential, 60% of extracts displayed LC_{50} value under 60 µg/mL and were identified to be extremely cytotoxic whereas 20% were categorized as discreetly cytotoxic (LC_{50} value ≥60 but ≤200 µg/mL). 20% of residual extracts were

considered low cytotoxic with LC_{50} values >200 µg/mL Table 4. Among all tested extracts IRM was found to be the best cytotoxic with 33.54 ± 0.66 µg/mL demonstrating that the polar solvents that are highly effective in the extraction of cytotoxic compounds as compared to non-polar solvents. DMSO in <1% working

Table 2: Antibacterial screening and Minimum inhibitory concentration of *I. rugosus* extracts against gram-positive and gram-negative bacteria.

Bacterial strains	200 µg/disc									
	IRM		IRE		IRC		IRA		IRH	
	ZOI	MIC	ZOI	MIC	ZOI	MIC	ZOI	MIC	ZOI	MIC
S. aureus ATCC 6538	34.5 ± 1.33	3.7	28.8 ± 1.3	11.11	13.1 ± 1.2	100	14.5± 2.42	100	----	----
B. subtilus ATCC 19659	30.12± 1.23	3.7	22.43± 1.53	33.33	----	----	12.54± 1.54	100	13.54± 2.54	100
P. aeruginosa ATCC 90271	21.16 ± 1.76	33.33	19.43± 1.76	100	28.43± 1.54	11.11	----	----	----	----
K. pneumonia ATCC 1705	33.33 ± 1.57	3.7	15.53± 1.76	100	34.5 ± 1.33	3.7	----	----	----	----
E. coli ATCC 33456	28.43± 1.53	11.11	24.54± 2.43	33.33	22.43± 1.43	33.33	----	----	----	----

ZOI zone of inhibition, MIC minimum inhibitory concentration, IRM: methanol, IRE: ethanol, IRC chloroform, IRA: ethyl acetate, IRH: n-hexane extract of *I. rugosus*.

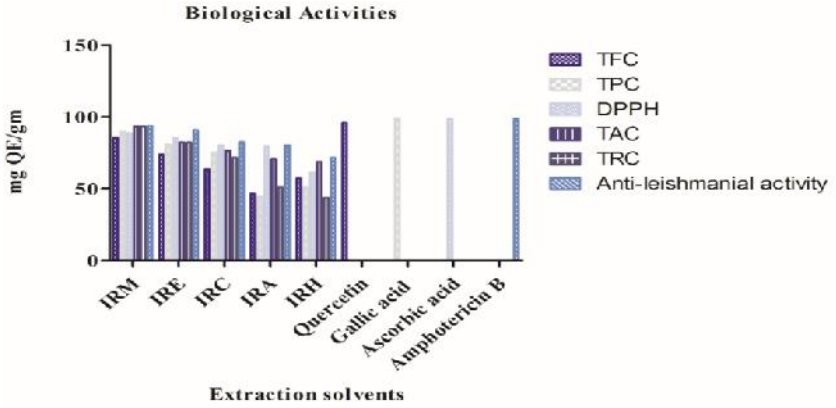

Figure 1: Total phenolic and total flavonoid content, DPPH, Total antioxidant capacity and total reducing power antioxidant assay, Anti-leishmanial activity on different extracts of *I. rugosus*. IRM: methanol, IRE: ethanol, IRC: chloroform, IRA: ethyl acetate, IRH: n-hexane extracts.

Table 3: Antifungal screening and Minimum inhibitory concentration of *I. rugosus* extracts against tested Fungal strains.

Fungal strains	200 µg/disc									
	IRM		IRE		IRC		IRA		IRH	
	ZOI	MIC	ZOI	MIC	ZOI	MIC	ZOI	MIC	ZOI	MIC
M. racemosus FCBP 0300	26.5 ± 1.21	33.33	22.7 ± 1.43	33.33	11.6 ± 1.81	100	----	----	----	----
C. albicans FCBP 478	20.2 ± 1.33	33.33	25.1 ± 1.11	33.33	12.53± 1.43	100	----	----	2.4±2.1	----
A. niger FCBP 0918	22.6 ± 1.63	33.33	17.5 ± 1.14	33.33	14.3 ± 1.42	100	3.5±2.4	----	----	----
F. solani FCBP 0291	18.2 ± 1.22	100	12.2 ± 1.26	100	16.1 ± 1.32	100	----	----	----	----
A. flavus FCBP 0064	20.3 ± 1.34	33.33	14.2 ± 2.21	33.33	20.4 ± 1.21	33.33	----	----	----	----

ZOI zone of inhibition, MIC minimum inhibitory concentration, IRM: methanol, IRE: *I* ethanol, IRC: chloroform, IRA: ethyl acetate, IRH: n-hexane extract.

concentration was used in the preparation of reaction mixture is considered safe in BSLT in comparison to Tween 20 (Wu, 2014). In the present study, 100% of the tested extracts showed LC_{50} values <1000 µg/mL signifying to be the presence of cytotoxic compounds that become responsible for the experimentally observed deaths. However, brine shrimp lethality activity in *Melia azedarach* methanol extract (Zahoor *et al*., 2015) is in strong agreement with our findings.

The results of the PKI assay in the measurement of the zones in mm were recorded for the *I. rugosus* extracts and are given in Table 4. A direct relationship was noticed amid concentrations of tested plant extracts and PKI activity. Amid all the tested samples, a significant bald area zone with ZOI 27 ± 1.35 mm and MIC = 11.11 µg/mL was measured around IRM followed by IRE with the bald zone (22 ± 1.52 mm) and IRC (18 ± 1.22 mm). Recently many researchers are taken interest in the protein kinase inhibition activity of plants. Phosphorylation of protein at tyrosine and serine/threonine residues by protein kinase is the major phenomena in the regulation of biological processes like metabolism, cell differentiation, apoptosis, and cell proliferation. Abnormal phosphorylation by protein kinase at tyrosine and serine/threonine residues results in genetic alterations that will further proceed in tumorigenesis and often become the cause of cancer. Therefore for cancer treatment, protein kinase inhibition plays a major role (Guangmin *et al*., 2011). Our findings are in settlement with Ahmed *et al* (2017) in which methanol extract of *Quercus dilatata* gives PKI activity.

Antidiabetic activity: Currently, there is an increase in interest towards the use of the traditional system of medicines used to cure different diseases including diabetes mellitus. Due to fact that herbal medicines have fewer side effects (Idm'hand *et al*., 2020). The alpha-amylase enzyme present in the human pancreatic small intestine and its activity is linked to surging in the level of sugar post-prandial levels, governing of this enzyme is, therefore, an important step for the treatment of type 2 diabetes caused by the deficiency in the secretion of insulin hormone or the human body becomes resistant to insulin (Justino *et al*., 2018). To analyze the antidiabetic effect of *I. rugosus* different extracts, alpha-amylase inhibition was used. IRH shows good activity with the value of 80.8 ± 2.9% alpha-amylase inhibition. After this extract, IRA & IRC with α-amylase inhibition of 76.7 ± 3.6% and 70.9 ± 2.4% respectively (Table 4). Our results were in good relation with the findings of Zahra *et al* (2017), discussing the antidiabetic activity of non-polar solvents.

Table 4: Brine shrimp lethality and Protein kinase inhibition assay.

Samples	Brine shrimp lethality (µg/mL)		Protein kinase inhibition (µg/mL)			Alpha-amylase (µg/mL)	
	% Mortality	LC_{50}	Diameter (mm) at 100 µg/disc		MIC	%age inhibition	IC_{50} µg/mL
	250		Clear Zone	Bald zone		250	
IRM	100 ± 1.80	33.54	----	29 ± 1.35 mm	11.11	50.7	112.5
IRE	91.5 ± 1.94	44.62	----	22 ± 1.52 mm	33.33	60.9	98.7
IRC	80.50 ± 1.80	53.3	----	18 ± 1.22 mm	100	70.3	60.7
IRA	60.5 ± 1.44	≤200	----	----	----	76.5	54.6
IRH	45.6 ± 1.5	>200	----	----	----	80.8	33.54

IRM methanol, IRE ethanol, IRC chloroform, IRA ethyl acetate, IAH n-hexane extract.

Conclusion: The current study conducted the first of its kind, attempted for thorough examination of micro-morphological traits and pharmacological evaluation regarding *I. rugosus*. LM and SEM study of pollen grains, seed, and epidermal micro-morphological characters has played a vital role in the identification of plants at species level. The biological & phytochemical outlining permitted the traditional uses of *I. rugosus* additionally also threw light on some important characteristics of the studied plant. This study suggested that the *I. rugosus* extracts are a good source of antioxidants augmented with phytochemicals, with elevated antimicrobial and protein kinase inhibitory properties. The best cytotoxic activity against brine shrimps was found in IRM. Anti-leishmanial and alpha-amylase inhibition potential was noticed to be more congregated in IRH. In this respect, additional characterization & isolation studies would have to be subjected to the assertion of the bioactive phytochemicals.

Acknowledgment: The authors thank full to the Department of Plant Sciences, Quaid-i-Azam University Pakistan for providing us with state-of-the-art research facilities.

Funding source: No specific funding was carried out for the carried research.

REFERENCES

Ahmad M., M.A. Khan, M. Zafar, M. Arshad, S. Sultana , B.H. Abbasi and S. Din (2010). Use of chemotaxonomic markers for misidentified medicinal plants used in traditional medicines. J. Med. Plant. Res 4(13): 1244-1252.

Ahmed M., H. Fatima, M. Qasim and Gul B (2017). Polarity directed optimization of phytochemical and in vitro biological potential of an indigenous folklore: *Quercus dilatata* Lindl. ex Royle. BMC Complem. Altern. M 17(1): 386-389.

Apu AS., S.H. Bhuyan, F. Khatun, M.S. Liza, M. Matin and M.F. Hossain MF (2013). Assessment of cytotoxic activity of two medicinal plants using brine shrimp (*Artemia salina*) as an experimental tool. Int. J. Pharm. Sci. Res 4(3): 1125-1129.

Ayaz, A., W. Zaman, F. Ullah, S. Saqib, S. Jamshed, S. Bahadur, A. Shakoor, B. Arshad. (2020). Systematics study through scanning electron microscopy; a tool for the authentication of herbal drug *Mentha suaveolens* Ehrh. Microsc Res Tech. 83(1), 81-87.

Chang CC., M.H. Yang, H.M. Wen and J.C. Chern (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J. Food. Drug. Anal 10(3): 121-127.

Chaves N., A. Santiago and J.C. Alias (2020). Quantification of the antioxidant activity of plant extracts: analysis of sensitivity and hierarchization based on the method used. Antioxidants 9(1): 76-83.

Csepregi R., V. Temesfoi, S. Das, A. Alberti, C.A. Toth, R, Herczeg, N. Papp and T. Koszegi (2020). Cytotoxic, antimicrobial, antioxidant properties and effects on cell migration of phenolic compounds of selected Transylvanian medicinal plants. Antioxidants 9(2): 166-172.

Goncalves GA., A.R. Spillere, das Neves, GM, L.P. Kagami, G.L. von Poser, R.F.S. Canto and V.L. Eifler-Lima (2020). Natural and synthetic coumarins as antileishmanial agents: A review. Eur. J. Med. Chem. 23(2): 112514-112519.

Guangmin Y., F.M. Sebisubi, L.Y.C. Voo, C.C. Ho, G.T Tan and L.C. Chang (2011). Citrinin derivatives from the soil filamentous fungus *Penicillium* sp. H9318. J. Braz. Chem. Soc 22(6): 1125-1129.

Gul S., M. Ahmad,M. Zafar M,S. Bahadur, S. Sultana , N. Begum, S.N. Shah, W. Zaman, F. Ullah, and A. Ayaz (2019). Taxonomic study of subfamily Nepetoideae (Lamiaceae) by polynomorphological approach. Microsc. Res. Tech 82(7): 1021-1031.

Gupta K., S. Maurya, S. Agarwal, A. Kushwaha and R. Kumar (2016). Antioxidant assessment of extracts obtained through hot extraction process. J. Mol. Cell. Biol 62(7): 129-132.

Hameed A., M. Zafar, R. Ullah, A.A. Shahat, M. Ahmad, S,I Cheema, I.U. Haq, S. Sultana, A. Usma and S. Majeed (2020). Systematic significance of pollen morphology and foliar epidermal anatomy of medicinal plants using SEM and LM techniques. Microsc. Res. Tech 83(8): 1007-1022.

Howlader MMS.,S.R. Ahmed, K. Kubra and M.K.H. Bhuiyan (2016). Biochemical and phytochemical evaluation of *Stevia rebaudiana*. Asian. J. Med. Biol. Res 2(1): 121-130.

Idm'hand E., F. Msanda,] and K. Cherifi (2020). Ethnopharma\cological review of medicinal plants used to manage diabetes in Morocco. Clin. Phytoscience 6: 1-32.

Islam E., R. Islam, Rahman, A.A, K. Alam, P. Khondkar , M. Rashid and S. Parvin (2013). Estimation of total phenol and in vitro antioxidant activity of *Albizia procera* leaves. BMC Res. Notes 6(1): 121-128.

Janbaz K.H., J. Arif, F. Saqib, I. Imran, M. Ashra, M. Zia-Ul-Haq, H.Z. Jaafar and V. De Feo (2014). In-vitro and in-vivo validation of ethnopharmacological uses of methanol extract of *Isodon rugosus* Wall. ex Benth.(Lamiaceae). BMC Complement. Altern. Med 14(1): 71-82.

Justino AB., N.C. Miranda, R.R. Franco, M.MM Martins, N.M. da Silva and F.S. Espindola (2018). *Annona muricata* Linn. leaf as a source of antioxidant compounds with in vitro antidiabetic and inhibitory potential against α-amylase, α-glucosidase, lipase, non-enzymatic glycation and lipid peroxidation. Biomed. Pharmacother 100(3):83-92.

Karaman I., F. Sahin , M. Gulluce, H. Ogutcu, H. Sengul and A. Adıguzel (2003). Antimicrobial activity of aqueous and methanol extracts of *Juniperus oxycedrus* L. J. Ethnopharmacol 85(2): 231-235.

Khalil AT., M. Ovais, I. Ullah, M. Ali, Z.K. Shinwari, D. Hassan and M. Maaza (2018). *Sageretia thea* (Osbeck.) modulated biosynthesis of NiO nanoparticles and their in vitro pharmacognostic, antioxidant and cytotoxic potential. Artif .Cells Nanomed. Biotechnol 46(4): 838-852.

Khan A., M. Suleman ,S. Abdul Baqi and M. Ayub (2019). Phytochemicals and their role in curing fatal diseases: A Review. Pure. Appl. Biol 8(1): 343-354.

Khan I., M.M. Yasinzai, Z. Mehmood, I. Ilahi, J. Khan, A. Khalil, M.S. Saqib and W.U. Rahman (2014). Comparative study of green fruit extract of *Melia azedarach* Linn. with its ripe fruit extract for antileishmanial, larvicidal, antioxidant and

cytotoxic activity. Am. J. Phytomed. Clin. Ther 2(3): 442-454.

Khan S., C.N.T. Taning, E. Bonneure, S. Mangelinckx, G. Smagghe, R. Ahmad, N. Fatima, M. Asif and M.M. Shah MM (2019). Bioactivity-guided isolation of rosmarinic acid as the principle bioactive compound from the butanol extract of *Isodon rugosus* against the pea aphid, *Acyrthosiphon pisum*. PloS one 14(6): 215048-215052.

Luqman M., M. Zafar, M. Ahmad, M. Ozturk, S. Sultana, F. Alam and F. Ullah (2019). Micromorphological observation of seed coat of *Eucalyptus* species (Myrtaceae) using scanning electron microscopy technique. Microsc. Res. Tech 82(2): 75-84.

Mahboubi A., J. Asgarpanah, P.N. Sadaghiyani and M. Faizi (2015). Total phenolic and flavonoid content and antibacterial activity of *Punica granatum* L. var. *pleniflora* flowers (Golnar) against bacterial strains causing foodborne diseases. BMC Complement. Altern. Med 15(1): 366-372.

Maina EN., V.N. Njau and Y. Gavamukulya (2020). Phytochemical analysis and antileishmanial activity of *Clerodendrum myricoides* and *Salvadora persica* Plant extracts against leishmania major. BMC Complement. Altern. Med 32(3): 29-44.

Malik K., M. Ahmad, M. Zafar, R. Ullah, H.M. Mahmood, B. Parveen, N. Rashid, S. Sultana and S.N. Shah (2019). An ethnobotanical study of medicinal plants used to treat skin diseases in northern Pakistan. BMC Complement. Altern. Med 19(1): 210-218.

Martinez-Morales F., A.J. Alonso-Castro, J.R. Zapata-Morales, C. Carranza-Alvarez and O.H. Aragon-Martinez (2020). Use of standardized units for a correct interpretation of IC50 values obtained from the inhibition of the DPPH radical by natural antioxidants. Chemical Papers 74(10): 3325-3334.

Mbagwu E., and H. Edeoga (2007). Morphology of *Vigna* species (Leguminosae) Medwell. Agricul. J 1(2), 200-205.

Mitra S., and R. Kannan (2007). A note on unintentional adulterations in Ayurvedic herbs. Ethnobot. Leafl 2007(1): 3-12.

Muhammad S., S. Ahmed, A. Ali, H. Huang, X. Wu, X.F. Yang, A. Naz and J. Chen (2014). Prioritizing drug targets in *Clostridium botulinum* with a computational systems biology approach. Genomics 104(1): 24-35.

Munir M., M. Ahmad, A. Waseem, M. Zafar, M. Saeed, A. Wakeel, M. Nazish and S. Sultana (2019). Scanning electron microscopy leads to identification of novel nonedible oil seeds as energy crops. Microsc. Res. Tech 82(7): 1165-1173.

Naz S., M. Zafar, M. Ahmad, R.A. Memon, S. Sultana, S. Bahadur, F.A. Ozdemir, Z. Siddiq and M.A. Shah (2019). Palynological investigation of lactiferous flora (Apocynaceae) of District Rawalpindi, Pakistan, using light and scanning electron microscopy. Microsc. Res. Tech 82(9): 1410-1418.

Neelamkavil SV., and J.E. Thoppil (2017). Chromosome Complements of *Isodon* (Family: Lamiaceae–Subfamily: Nepetoideae): Trends in Karyotype Evolution. Proceedings of the National Academy of Sciences, India Section B: Bio. Sci. 87(2): 267-272.

Nguyen M., V. Nguyen, L. Minh, L. Trieu, M. Cang, L. Bui, X. Le and V. Danh (2020). Determination of the phytochemical screening, total polyphenols, flavonoids content, and antioxidant activity of soursop leaves (*Annona muricata* Linn.), IOP Conference Series: Materials Science and Engineering. IOP Publishing: 2011.

Perveen A., and M. Qaiser (2004). Pollen morphology of the family Labiatae from Pakistan. Pakistan J. Bot. 35(5): 671-694.

Phull AR., Q. Abbas, A. Ali, H. Raza, M. Zia and Haq Iu (2016). Antioxidant, cytotoxic and antimicrobial activities of green synthesized silver nanoparticles from crude extract of *Bergenia ciliata*. Future. J. Pharm. Sci 2(1): 31-36.

Rashid S., M. Ahmad, M. Zafar, S. Sultana, S.N. Ahmad, G. Yaseen, F.A. Ozdemir and R. Afza (2019). Microscopic and phytochemical techniques as a tool for authentication of herbal drug chiraita: *Swertia cordata* (G. Don) CB Clarke. Microsc. Res. Tech 82(7): 1092-1101.

Ruwali P., and D. Negi (2019). Phytochemical analysis and evaluation of antioxidant activity of *Premna latifolia* Roxb. A medicinal plant (Family: Lamiaceae). J. Pharm. Innov 8(5): 13-20.

Sadia H., M. Zafar, M. Ahmad, M.P. Khan, G. Yaseen, M.I. Ali, S. Sultana, O. Kilic, Z. Sahan and N. Alam (2019). Foliar epidermal anatomy of some selected wild edible fruits of Pakistan using light microscopy and scanning electron microscopy. Microsc. Res. Tech 32(3):43-48.

Sadiq A., A. Zeb, S. Ahmad, F. Ullah, M. Ayaz, F. Ullah, N. Ali, J. Ahmad and F.A. Khan (2019). 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. Pakistan J. Pharm. Sci 32(5): 32-38.

Sadiq A., A. Zeb, F. Ullah, S. Ahmad, M. Ayaz, U. Rashid and N. Muhammad (2018a). Chemical

characterization, analgesic, antioxidant, and anticholinesterase potentials of essential oils from *Isodon rugosus* Wall. ex. Benth. Front. Pharmacol 9(3): 623-632.

Sadiq A., A. Zeb, F. Ullah, S. Ahmad, M. Ayaz, U. Rashid and N. Muhammad (2018b). Chemical characterization, analgesic, antioxidant, and anticholinesterase potentials of essential oils from *Isodon rugosus* Wall. ex. Benth. Front. Pharmacol 9(3): 32-38.

Sahreen S., M.R. Khan and R.A. Khan (2014). Comprehensive assessment of phenolics and antiradical potential of *Rumex hastatus* D. Don. roots. BMC Complement. Altern. Med 14(1): 47-54.

Siddiquah A., S.S. Hashmi, S. Mushtaq, S. Renouard, J.P. Blondeau, R. Abbasi, C. Hano and B.H. Abbasi (2018). Exploiting in vitro potential and characterization of surface modified Zinc oxide nanoparticles of *Isodon rugosus* extract: Their clinical potential towards HepG2 cell line and human pathogenic bacteria. Excli. J 17(8): 671-683.

Sufyan M., I. Badshah, M. Ahmad, M. Zafar, S. Bahadur and N. Rashid (2018). Identification of medicinally used Flora using pollen features imaged in the scanning electron microscopy in the lower Margalla Hills Islamabad Pakistan. Microanal 24(3): 292-299.

Talbi S., J.A. Rojas, M. Sahrawy, M. Rodriguez-Serrano, K.E. Cardenas, M. Debouba and L.M. Sandalio (2020). Effect of drought on growth, photosynthesis and total antioxidant capacity of the saharan plant *Oudeneya africana*. Environ. Exp. Bot 176(8): 104089-104099.

Wen L., X. Guo, R.H. Liu, L. You, A.M. Abbasi and X. Fu (2015). Phenolic contents and cellular antioxidant activity of Chinese hawthorn "*Crataegus pinnatifida*". Food Chem 186: 54-62.

Wu C., (2014). An important player in brine shrimp lethality bioassay: The solvent. J Adv Pharm Technol Res. 5(1):57-59.

Yao G., F.M. Sebisubi, Voo LYC, C.C. Ho, G.T. Tan and LC. Chang (2011). Citrinin derivatives from the soil filamentous fungus *Penicillium* sp. H9318. J. Braz. Chem Soc 22(6): 1125-1129.

Zahoor M., M. Ahmed, S. Naz and M. Ayaz (2015). Cytotoxic, antibacterial and antioxidant activities of extracts of the bark of *Melia azedarach* (China Berry). Nat. Prod. Res 29(12): 1170-1172.

Zahra SS., M. Ahmed, M. Qasim, B. Gul, M. Zia, B. Mirza and Haq Iu (2017). Polarity based characterization of biologically active extracts of *Ajuga bracteosa* Wall. ex Benth. and RP-HPLC analysis. BMC Complement. Altern. Med 17(1): 443-446.

Zeb A, Sadiq A., F. Ullah, S. Ahmad and M. Ayaz (2014). Phytochemical and toxicological investigations of crude methanolic extracts, subsequent fractions and crude saponins of *Isodon rugosus*. Biol. Res 47(1): 57-62.

Zeb A., F. Ullah, M. Ayaz, S. Ahmad and A. Sadiq (2017). Demonstration of biological activities of extracts from *Isodon rugosus* Wall. Ex Benth: Separation and identification of bioactive phytoconstituents by GC-MS analysis in the ethyl acetate extract. BMC Complement Altern. Med 17(1): 284-291.