Anti-Leishmanial Evaluation of *Fraxinus xanthoxyloides* (G. Don) DC. Collected from District Islamabad

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Summary: In this study, we have investigated phytochemical classes, antileishmanial and cytotoxic activity of *Fraxinus xanthoxyloides* (Oleaceae) leaves. Powder of *F. xanthoxyloides* leaves was extracted with methanol to obtain the crude extract (FXM) and the resultant was fractionated with solvents in escalating polarity; *n*-hexane (FXH), chloroform (FXC), ethyl acetate (FXE), *n*-butanol (FXB) and the residual aqueous (FXA) fraction. Quantitative estimation of terpenoids, coumarins, flavonoids, phenolics and tannins was conducted. Anti-leishmanial activity was performed against *Leishmania tropica* promastigote stage parasite while insecticidal activity was assessed through brine shrimps lethality assay. Our results showed the maximum concentration of terpenoids in FXC while the highest quantity of coumarins, flavonoids, phenolics and tannins was recorded in FXE. Presence of terpenoids was not detected in FXB and in FXA. Among the extract/fractions, FXC exhibited the highest anti-leishmanial activity with LD_{50} of 15.23±0.9 µg/ml to that of glucantime (LD_{50} = 5.6±2.4 µg/ml) a reference drug. FXH exhibited the anti-leishmanial activity of LD_{50} = 40.68±1.9 µg/ml followed by FXE (LD_{50} = 102.9±3.1 µg/ml). Similarly potent insecticidal activity was recorded (LD_{50} = 28.15±1.8 µg/ml) for FXC followed by FXH (LD_{50} = 67.59±2.3 µg/ml). However, other fractions exhibited low level of anti-leishmanial and insecticidal activity. Correlation analysis exhibited a strong association ($p < 0.05$) between the terpenoids and the anti-leishmanial activity and a second but non significant association ($p > 0.05$) with the insecticidal activity. The coumarins established a medium association with the insecticidal activity. Other chemical classes exhibited a moderate to low level of association with the anti-leishmanial and the insecticidal activity. On the basis of these results we can conclude that chloroform fraction of *F. xanthoxyloides* is a potential source for anti-leishmanial and insecticidal activities and further studies are required to isolate the active constituents.

Keywords: *Fraxinus xanthoxyloides;* Phytochemical; Anti-leishmanial; Cytotoxicity; Brine shrimp.

Introduction

Extensive evaluation of medicinal plants and their folk uses has been carried out in last 40-50 years research. World Health Organization has nominated 252 drugs as “essential drugs” of which 11 % are derived from the natural products [1]. Plant kingdom being an excellent source of easily isolated drugs has earned domination to herbal products being safer, cost effective and with reduced side effects than that of synthetic compounds [2].

The activities of plants extract against different infections may reside in the variety of different structures, essential oils, phenolics and aldehyde compounds [3]. The secondary metabolites i.e. tannins, terpenoids, coumarins, alkaloids, phenolic compounds, flavonoids present in plants show variety of biological activities like antioxidant, cytotoxic as well as antiparasitic potential [3, 4].

Leishmaniasis, is a group of diseases the causative agent of which belong to several species of *Leishmania* exhibiting different forms of clinical symptoms. It is the largest occurring disease among the vector borne diseases after flariasis and malaria [5]. WHO categorized leishmaniasis as a category 1 disease, i.e. uncontrollable and emerging [6]. Three clinical types of leishmaniasis has been recognized; visceral, cutaneous and mucocutaneous leishmaniasis. Visceral leishmaniasis accounts 500,000 new cases each year in 61 countries and among these 90% cases belong to India, Bangladesh, Nepal, Sudan, and North Eastern Brazil [7].

In Pakistan leishmaniasis was first accounted in northern areas in 1960. In the beginning it was restricted to northern sphere but now it is widely spreading throughout the country [8]. Both cutaneous and visceral leishmaniasis is the major threats to Pakistan despite of, mucocutaneous leishmaniasis which is rarely reported [4]. *Leishmania tropica* is the causative agent of cutaneous leishmaniasis and is accompanied by long lasting ulcers on stratum cornium of skin tissue, which results in patient’s disgrace and humiliation in society. Cutaneous leishmaniasis is a vector borne

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disease and is transmitted from person to person by a third party; sandfly. The infection is readily disseminated within a specific area depending upon several factors like population density, poor hygiene, improper sanitation and lack of protection from blood sucking insects like mosquito, flee and lice [9].

Issues of toxicity and resistance is therefore compelling us to ponder and work on a new therapy for this disease which is clinically more efficacious than currently available medication, have milder side effects and is cost effective. So scientists are working hard to develop such drugs from plant sources that are easily available and have low production cost.

Cancer is a leading cause of death all over the world, about 12 million people all over the world are diagnosed cancer each year and it kills more people than tuberculosis, malaria and AIDS collectively. Among various bioassays to assess antitumor activity brine shrimp lethality assay mostly used to screen the plant extracts/fractions or compounds, because of strong association of the human nasopharyngeal carcinoma with brine shrimp toxicity. It provides fundamental guidelines to isolate active compounds [10]. This method is practiced quite often to assess possibility of anticancer activity of plant extracts and then develop new anticancer drugs. Already a number of cytotoxic and pesticidal agents are developed through this system. Screening of medicinal plants for phytochemicals and antiparasitic activities is important for finding the potential raw compounds for therapeutic use. In our study we have selected *Fraxinus xanthoxyloides* for quantitative determination of secondary metabolites, anti-leishmanial and insecticidal activities.

Experimental

Plant Collection

The leaves of *F. xanthoxyloides* were collected in October, 2013 from the campus of Quaid-i-Azam University Islamabad, Pakistan. The plants were recognized by their local names and then validated by Rizwana Aleem Qureshi, Professor, Plant Sciences Department, Quaid-i-Azam.University Islamabad, Pakistan. Specimen (45679) was submitted to National Herbarium, Quaid-i-Azam.University, Islamabad.

Extract Preparation

After collection, plant samples were shade dried till the complete removal of moisture and samples were made to mesh sized powder by using plant grinder. Powder (2kg) of the sample was soaked in crude methanol (4L) for extraction for 72 h. All the samples were processed two times repeating above procedure. For the purpose of filtration, Whatman No. 1 filter was used and methanol was evaporated on a rotary evaporator at 40°C under reduced pressure. To sort the compounds in the crude extract with increasing polarity, crude extract (30g) was suspended in distilled water (250ml) and passed to liquid-liquid partition by using solvents as *n*-hexane (FXH), chloroform (FXC), ethyl acetate (FXE), *n*-butanol (FXB) and aqueous (FXA) fractions. Rotary evaporator was used to concentrate the fraction by evaporating the solvent under reduced pressure at 40°C [4]. Extracts and fractions were dried and then stored at 4°C for the investigation of phytochemical constituents, insecticidal and anti-leishmanial activities.

Quantitative Estimation of Secondary Metabolites

Determination of Terpenoids

An aliquot of 1.5ml of chloroform was mixed with 100µl of extract/fraction (10mg/ml). After 3 min of mixing 100µl of conc. sulphuric acid (H_2SO_4) was added and the assay tubes were incubated for 5 min. Reddish brown precipitate formed was dissolved in 1.5ml of 95% methanol. The spectrophotometer was adjusted to a 538 nm wavelength to measure the absorbance. The test was performed in at least triplicate and the total terpenoid content was expressed as milligrams of linalool equivalents per gram of the sample extract (mg LE/g) [11].

Determination of Coumarins

To quantify the coumarins, 500µl (10mg/ml) of the extract/fractions was transferred to a test tube followed by the addition of 2ml of distilled water and 500µl of 1% lead acetate. After thorough shaking, 7ml of distilled water was added to the reaction mixture. A volume of 2ml of the reaction mixture was mixed with 8ml of hydrochloric acid (1N) and allowed to stand at room temperature for 30 min and absorbance was recorded at 320 nm. The test was performed in triplicate and the total coumarin content was expressed as milligrams of coumarin equivalents (1,2-benzopyrone) per gram of the sample extract (mg CE/g) [12].

Determination of Total Flavonoids

Flavonoid content was determined following Park *et al*. [13] with some modifications. In a 10ml test tube, 500µl (10mg/ml) of crude extract and fractions, 3.4ml of 30% methanol, 150µl of 5% sodium nitrite ($NaNO_2$) and 300µl of 10% aluminium chloride hexahydrate ($AlCl_3.6H_2O$) were added and mixed. After 5 min, 1ml of 1M sodium hydroxide (NaOH) was added and allowed to stand for 5 min.

The absorbance was measured at 510 nm by using UV/VIS-DAD spectrophotometer. The standard curve for total flavonoids was made using rutin standard solution (6.25-100 µg/ml) under the same procedure as above. The total flavonoids were expressed as milligrams of rutin equivalents per gram (mg RE/g) of dried sample.

Determination of Total Phenolics

The total phenolics were determined in the extract/fractions by the spectrophotometric method [14]. In brief, a 300µl of the test sample was transferred to test tube and 2.25ml of Folin-Ciocalteu reagent (previously diluted with distilled water by 10 fold) was added and then mixed. The resulting mixture was allowed to stand at room temperature for 5 min. Then 2.25ml of 6% (w/v) sodium carbonate was added to above mixture. The resulting mixture was placed for 90 min at room temperature and absorbance was taken at 725 nm by using UV/VIS-DAD spectrophotometer. The standard curve for total phenolics was made using gallic acid standard solution (6.25-100µg/ml) under the same procedure as above. The total phenolics were expressed as milligrams of gallic acid equivalents per gram of dried sample.

Determination of Tannins

For the quantitative determination of tannins polyvinyl polypyrrolidone (PVPP) assisted quantification of tannins was done [15]. A test tube containing 100mg of PVPP was mixed with 1ml of distilled water followed by 1ml of test sample (10mg/ml). After 4 h of reservation at 4°C the reaction mixture was thoroughly mixed and centrifuged (2000 × g for 10 min at room temperature) to get bilayer mixture. PVPP causes the precipitation of tannins while supernatant contains the phenolics other than tannins. Then phenolic contents were determined as above [14] in the supernatant as non-tannin phenolics. Tannins are then calculated by this formula:

Tannin = Total phenolics – non-tannin phenolics

Anti-Leishmanial Assay

Stock solution for anti-leishmanial assay was prepared by dissolving 5mg/ml of each plant extract and derived fractions in 1ml of DMSO. Stock solutions were further diluted serially (1000, 700, 500, 50, 5, 0.5 and 0.05µg/ml) using DMSO to obtain the appropriate concentrations. Samples were filtered by using a 0.45µm syringe filter. *Leishmania tropica* KWH23 was previously isolated from a patient in Peshawar, Pakistan and was characterized (data not shown). The promastigotes form of the *Leishmania* were grown in M199 medium with 10% Fetal calf serum (FCS), HEPES buffer, streptomycin and penicillin. Log phase promastigotes at 1×10^6/100µl were used for the entire assay. About 90µl of 199 media, 50µl of *Leishmania tropica* KWH23 log phase culture and 10µl of each plant dilution was dispensed to different wells of micro-titter plate. Here, DMSO (10µl) was used as a negative control, while glucantime as positive control. Afterwards, micro-titter plate was incubated at 24°C for 72 h. After incubation about 15µl of each dilution was pipetted on a neubauer counting chamber and were counted under a microscope [4].

Insecticidal Activity

To estimate the toxicity of the plant extracts and their fractions, brine shrimp lethality assay was used. For the hatching of brine-shrimps (*Artemia salina*) eggs, at an ambient temperature of 23 ± 1°C artificial sea water (3.8g sea salt/L) was used [3]. Three different concentrations of each extract (1000, 100 and 10µg/ml) were made, taken from 10mg/ml stock solution in methanol. Methanol was evaporated before transferring shrimps to the vials. After 24 h, the hatched shrimps were shifted to the vials filled with 5ml of artificial sea water (10 shrimps per vial) along with samples. As a positive control, from stock solution of tricaine methanesulfonate (1mg/ml) serial dilutions were made in the range of 0.05 µg/ml – 100µg/ml in DMSO. The number of the shrimps that survived the sample environment was counted after 24 h.

Cytotoxicity Studies

Characterization of extract/fractions for cytotoxicity was demonstrated according to You *et al*. [15] by the sulforhodamine B assay. Briefly, 190 µl of RAW 264.7 (ATCC-TIB-71) cells with a density of 5×10^4 cells/ml were seeded in 96-well plate having 10 µl of the test sample (final concentration of 20 µg/ml) in DMSO (10%) and PBS, and incubated for 72 h at 37 ºC in CO_2 incubator. After incubation 50 µl of 20% TCA was added to terminate the reaction. Cells were washed, dried and stained with 0.4% of acetic acid for 30 min at room temperature. Cells were washed four times with acetic acid and dried overnight. Bound dye was solubilized in 200 µl of 10 mM of Tris base (pH:10) on a gyratory shaker for 10 min. Absorbance of each treatment was recorded at 515 nm through a micro-plate reader. A zero-day control was performed in each case following addition of equal quantity of cells in sixteen wells, with subsequent incubation for 30 min at 37 ºC and was processed as mentioned earlier. Cell survival percentage was calculated for each test sample.

Statistical Analysis

GraphPad Prism software version 5.0 (2007) was used to calculate LD_{50} values of each sample for brine shrimp toxicity assay and anti-leishmanial activity. Nonlinear regression test was used to determine R^2, and LD_{50} values. Correlation between the LD_{50} value of the anti-leishmanial and insecticidal activity with the chemical class was established by using the Statistix 8.1.

Results and Discussion

In order to cure different diseases, the residents of Pakistan belonging to rural areas are dependent on the indigenous system of medicine. On the basis of conventional use of plants, we carried out the *in vitro* biological screening of crude methanol extract and its derived fractions of *F. xanthoxyloides* leaves. It is well acknowledged, that the medicinal value of plants is due to the presence of bioactive phyto-components [16]. Hence the study of plant constituents is helpful to explain the traditional uses of plants. In this investigation crude methanol extract and fractions of *F. xanthoxyloides* were investigated for the quantitative determination of terpenoids, coumarins, flavonoids, tannins and phenolics (Table-1). Medicinal plants have prime importance to community health and various pharmacological effects are produced due to the presence of plant secondary metabolites. Secondary metabolites like terpenoids, tannins and flavonoids have various beneficial roles to cure various human ailments.

The amount of phytochemicals which were found in extract/fractions of *F. xanthoxyloides* was determined by the standard procedures. Table-1 depicted the terpenoids content in extract/fractions of *F. xanthoxyloides* which were estimated as milligrams of linalool equivalents (LE) per gram of sample. In our study the highest terpenoid content was found in FXC (100.41mg/g) followed by FXM (73.16mg/g) > FXH (51.41mg/g) > FXE (17.75mg/g) while in FXB and FXA terpenoids were not present. Terpenoids have earned a reputation as anti-inflammatory, analgesic, hepatoprotective, antipyretic, cardiotonic, general tonic and sedative agent in many Asian countries [17,18]. Recent studies added more belief that terpenoids are not only capable of above mentioned pharmacological properties but also have antioxidant, antiviral, antiangiogenic, antimicrobial, anti allergic, antipyretic and spasmolytic aptitude [19- 21]. An admiring number of triterpenoids have been reported to possess selective cytotoxic ability against a range of cancer cells without any harmful effect to the normal cells [22, 23].

Total coumarin contents were estimated in terms of 1,2-benzopyrone equivalents (BPE) which are presented in Table-1. Coumarin contents were high in FXE (60.20mg BPE/g sample) as compared to other fractions and the lowest amount was observed in FXH (0.40mg BPE/g sample). Coumarins are lactones of O-hydroxy-cinnamic acid derived from trans-cinnamic acid via oxidation-reduction and isomerization to produce 1,2-benzopyrone. Coumarins can be categorized into simple coumarins, pyranocoumarins, furanocoumarins, chromones and dimeric coumarins. Coumarins have antioxidant, antispasmodic, spasmolytic, anti-HIV, hypolipidemic, vasodilating and hypotensive capacities [12].

Total flavonoid content (TFC) was calculated as rutin equivalent (RE) and was varied noticeably in the extract/fractions of *F. xanthoxyloides* (Table-1). This study showed the highest TFC in FXE (44.04mg RE/g sample), followed by FXB (43.37mg RE/g sample) > FXM (41.70mg RE/g sample) > FXC (34.76mg RE/g sample) > FXA (9.70mg RE/g sample) > FXH (2.82mg RE/g sample). According to reports the phenolics can be scrutinized into several classes, of which flavonoids being the major one have potential antioxidant capabilities [17]. Naturally occurring secondary metabolites in plants like flavonoids are quite beneficial to human health. Studies have affirmed the antibacterial, anti-inflammatory, antiviral, anticancer and anti-allergic aptitude of flavonoids derivatives [24, 25].

Table-1: Quantitative screening of phytochemicals in *F. xanthoxyloides.*

Extract/ Fraction	Total terpenoids mg/g dry sample	Total coumarins mg BPE/g dry sample	Total flavonoids mg RE/g dry sample	Total phenolics mg GAE/g dry sample	Total tannins mg GAE/g dry sample
FXM	$73.16^{b}\pm3.2$	$37.00^{b}\pm2.9$	$41.7^{a}\pm5.8$	$262.74^{a}\pm2.1$	$46.80^{b}\pm2.9$
FXH	$51.41^{c}\pm2.7$	$0.40^{f}\pm0.7$	$2.82^{d}\pm1.1$	$202.22^{e}\pm4.2$	$1.20^{d}\pm0.8$
FXC	$100.41^{a}\pm5.6$	$25.40^{d}\pm1.8$	$34.76^{b}\pm2.2$	$203.08^{e}\pm3.9$	$79.20^{a}\pm3.2$
FXE	$17.75^{d}\pm1.1$	$60.20^{a}\pm3.4$	$44.04^{a}\pm4.9$	$256.36^{b}\pm2.7$	$81.40^{a}\pm3.8$
FXB	0^{e}	$28.00^{c}\pm2.2$	$43.37^{a}\pm4.1$	$241.53^{c}\pm2.9$	$46.60^{b}\pm2.4$
FXA	0^{e}	$11.80^{e}\pm1.4$	$9.7c^{c}\pm0.9$	$206.87^{d}\pm3.2$	$33.40^{c}\pm1.7$

Mean ± SE (n=3).

FXM; *F. xanthoxyloides* methanol extract, FXH; *F. xanthoxyloides n*-hexane fraction, FXC; *F. xanthoxyloides* chloroform fraction, FXE; *F. xanthoxyloides* ethyl acetate fraction, FXB; *F. xanthoxyloides n*-butanol fraction, FXA; *F. xanthoxyloides* aqueous fraction. The means not sharing common letter differ significantly at $p < 0.05$.

Table-2: Anti-leishmanial screening of various fractions of *F. xanthoxyloides*.

Extract/Fraction	Concentration (μg/ml)								
	1000	700	500	50	5	0.5	0.05	LD_{50} μg/ml	R^2
FXM	100	72±1.6	33±2.6	0	0	0	0	575.0±2.5	0.991
FXH	100	100	95±3.2	60±2.8	0	0	0	40.68±1.9	0.996
FXC	100	100	100	75±2.2	20±1.9	0	0	15.23±0.9	0.999
FXE	100	100	90±1.5	23±1.4	2±2.2	0	0	102.9±3.1	0.997
FXB	74±2.7	24±2.1	0	0	0	0	0	846.1±2.8	0.993
FXA	25±3.1	18±1.9	12±1.3	0	0	0	0	2314.0±3.6	0.999

Mean ± SE (n=3)

Glucantime used as standard drug expressed LD_{50} of 5.6μg/ml. FXM; *F. xanthoxyloides* methanol extract, FXH; *F. xanthoxyloides n*-hexane fraction, FXC; *F. xanthoxyloides* chloroform fraction, FXE; *F. xanthoxyloides* ethyl acetate fraction, FXB; *F. xanthoxyloides n*-butanol fraction, FXA; *F. xanthoxyloides* aqueous fraction.

Total phenolic contents (TPC) of all the extract/fractions were calculated in terms of gallic acid equivalents (TAE) as shown in Table-1. The present study showed the TPC in the subjected plant as: FXM (262.74mg TAE/g sample) > FXE (256.36mg GAE/g sample) > FXB (241.53mg GAE/g sample > FXA (206.87mg GAE/g sample) > FXC (203.08mg GAE/g sample) > FXH (202.22mg GAE/g sample). Because of improving quality, nutritional value of foods and impeding oxidative degradation of lipids, phenolics rich plant materials have earned a reputation [26]. Phenolics, the phenylalanine and tyrosine derivatives are considered as secondary metabolites which occur universally in plants and are diversified [27]. Table-1 presented the total tannin contents. We observed that tannins were high in FXE (81.4mg GAE/g sample) and FXC (79.2mg GAE/g sample) and the lowest amount was observed in FXH (1.2mg GAE/g sample) which was almost negligible. Wani *et al*. [28] screened the methanol, ethyl acetate and hexane extracts of *Hyptis spicigera* plant for qualitative and quantitative analysis of phytochemicals through standard procedures. Quantitative analysis showed the presence of terpenoids (16.10%), phenolics (20.75%), alkaloids (7.55%), flavonoids (8.82%) and saponins (6.23%). Methanol extract of *Gentiana kurroo* Royle (Gentianaceae) roots was screened by [29] and declared this important and omnipresent medicinal plant of Kashmir Himalaya to possess several bioactive metabolites and nociceptive activity. Tannins, saponins, cardiac glycosides, alkaloids, terpenes, carbohydrates, flavonoids and phenolics were found to be present on phytochemical evaluation.

Table-2 displays the LD_{50} values for anti-leishmanial activities of the crude methanol extract and its derived fraction of *F. xanthoxyloides*. It was recorded that chloroform fraction exhibited the best anti-leishmanial activity (LD_{50}=15.23±0.9μg/ml, R^2=0.999) whereas the least effective was the aqueous fraction which have displayed the highest LD_{50}=2314±3.6μg/ml, R^2=0.999 value. The LD_{50} value recorded for the standard drug glucantime was 5.6±2.4μg/ml, R^2=0.999. Anti-leishmanial activities of various plants have been reported in earlier studies. Leaf extract of *Anisomeles malabarica* and *Ricinus communis* exhibited good anti-leishmanial activity (LD_{50} = 126±19.70 and 184±39.33μg/ml), respectively [30]. Similarly Shah *et al.* [4] reported the anti-leishmanial activity of *Jurenia dolomiaea* roots against *Leishmania tropica* KWH23 promastigotes. The methanol extract of *J. dolomiaea* roots exhibited LD_{50} = 10.9±1.1μg/ml while its ethyl acetate fraction manifested LD_{50} = 5.3±0.2μg/ml) anti-leishmanial activity against *L. tropica*.

Anti-leishmanial activity of *F. xanthoxyloides* has been reported for the first time in this study. The treatments currently in use as prime therapy for leishmaniasis include meglumine antimoniate (Glucantime), in addition to pentavalent antimonials sodium stibogluconate (Pentostam), but they are harmful to some extent, require prolong parenteral administration courses and have powerful side effects [31]. In some cases, pentamidine as well as amphotericin B are used as second line treatment which may also have lethal effects [31]. The results of our study revealed pharmacological activity against *L. tropica* by *F. xanthoxyloides* and our data suggests that the chloroform fraction has the potential use for leishmaniasis. Further studies are required to investigate the active compounds responsible for anti-leishmanial activity and their *in vivo* application to sought out their appropriate concentration against *Leishmania* in infected experimental organisms. It would be helpful in designing new medicines which are biologically more active and cost-effective and having minimum side effects.

Insecticidal screening using brine shrimps bioassays was carried out to provide important preliminary data to screen plant extracts for their anti-cancerous potential. Insecticidal effect of crude extract and various fractions of *F. xanthoxyloides* were studied for insecticidal activity through BSLA under controlled conditions. Direct relation was found between brine shrimps lethality and concentration of different extracts/fractions (Table-

3). LD_{50} is measure of therapeutic index of a substance/extract. LD_{50} was the lowest concentration of test sample at which deaths of half of the shrimps took place. Data of present study indicated that the order of LD_{50} for *F. xanthoxyloides* was in the following order i.e. FXC > FXH > FXM > FXB > FXA > FXE. In case of insecticidal studies chloroform fraction of *F. xanthoxyloides* showed the best activity, (LD_{50} = 28.15±1.8µg/ml). The association between the LD_{50} value of the anti-leishmanial and insecticidal activity with chemical classes is given in Table-4. The results indicated significant ($p < 0.05$) correlation between the anti-leishmanial activity and the terpenoid constituents. Terpenoids also indicated a medium correlation with the insecticidal activity. The other classes such as the coumarins, phenolics, flavonoids and tannins provided a moderate association with the anti-leishmanial activity. However, low intensity of association between the insecticidal activities with these chemical classes was established in this study. The potential pharmacological effects of terpenoids have been investigated in earlier studies where terpenoids exhibited the selective aptitude towards the cells [23]. Therefore, the presence of terpenoids in the chloroform fraction can identify its use in various ailments of human beings.

Table-3: Insecticidal screening of various fractions of *F. xanthoxyloides* against brine shrimps after 24 h.

Extract/ Fraction	Concentration µg/ml				
	1000	100	10	LD_{50} µg/ml	R^2
FXM	60±1.5	45±1.4	25±2.1	245±1.1	0.9876
FXH	75±2.3	60±2.1	25±1.5	67.59±2.3	0.9601
FXC	90±1.6	60±2.5	40±2.3	28.15±1.8	0.9570
FXE	40±2.1	25±1.5	15±1.3	409±3.2	0.9995
FXB	65± 3.5	35±3.4	25±3.6	276.6±1.9	0.9461
FXA	60±1.2	35±1.7	15±1.7	404.6±2.2	0.9995

Mean ± SE (n=3)
Tricaine methanesulfonate used as standard expressed LD_{50} of 4.315 ± 2.2µg/ml. FXM; *F. xanthoxyloides* methanol extract, FXH; *F. xanthoxyloides n*-hexane fraction, FXC; *F. xanthoxyloides* chloroform fraction, FXE; *F. xanthoxyloides* ethyl acetate fraction, FXB; *F. xanthoxyloides n*-butanol fraction, FXA; *F. xanthoxyloides* aqueous fraction.

Table-4: Correlation between LD_{50} for anti-leishmanial and insecticidal activity with the chemical class.

	Correlation	
	Anti-leishmanial activity (LD_{50})	Insecticidal activity (LD_{50})
Terpenoids	0.8617*	0.6624
Coumarins	0.6154	0.1572
Flavonoids	0.5794	0.2049
Phenolics	0.3895	0.1255
Tannins	0.5536	0.2119

* Indicate significant correlation at $p < 0.05$ between the chemical class and the biological activity.

Table-5 indicated the cytotoxic effects of *F. xanthoxyloides* extract/fractions on RAW 264.7 cells (ATCC-TIB-71) cells during *in vitro* studies. All the extract and fractions did not cause cytotoxicity and greater than 90% survival was determined for the tested samples. In view of these results it can be established that plant is non toxic at 20µg/ml to these cells.

Table-5: Cytotoxicity assay for *F. xanthoxyloides* extract/fractions used in the *in vitro* model.

Sample	Survival (%)
FXM	97.8±2.9
FXH	104.6±9.0
FXC	101.1±8.74
FXE	105.2±6.7
FXB	97.0±10.5
FXA	91.0±0.1

Values are expressed as mean±SD (n = 3).

Conclusion

It can be concluded that, *F. xanthoxyloides* is an asset for the field of ethnopharmacology as it is used traditionally in various regions of Pakistan to solve different health related problems. Therefore, there is a need to isolate bioactive compounds from this plants which may be potential source for their anti-leishmanial and insecticidal activities.

Competing Interest: The authors declare no competing interest.

References

1. S. M. K. Rates, Plants as Source of Drugs, *Toxicon.,* **39**, 603 (2001).
2. R. Yadav and M. Agarwala, Phytochemical Analysis of Some Medicinal Plants, *J. Phytol.,* **3**, 12 (2011).
3. S. Sahreen, M. R. Khan, R. A. Khan and N. A. Shah, Estimation of Flavoniods, Antimicrobial, Antitumor and Anticancer Activity of *Carissa opaca* Fruits, *BMC Compl. Alter.Med.,* **13**, 372 (2013).
4. N. A. Shah, M. R. Khan, K. Naz and M. A. Khan, Antileishmanial, Toxicity, and Phytochemical Evaluation of Medicinal Plants Collected from Pakistan, *Biomed Res. Inter.,* **2014**, 726241 (2014).
5. L. Solano Gallego, A. Koutinas, G. Miró, L. Cardoso, M. Pennisi, L. Ferrer, P. Bourdeau, G. Oliva, G. Baneth, Directions for the Diagnosis, Clinical Staging, Treatment and Prevention of Canine Leishmaniosis, *Vet. Parasitol.,* **165**, 1 (2009).
6. H. W. Murray, J. D. Berman, C. R. Davies, N. G. Saravia, Advances in Leishmaniasis, *The Lancet,* **366,** 1561 (2005).
7. World Health Organization, *Report of the Fifth Consultative Meeting on Leishmania/HIV*

Coinfection. Addis Ababa, Ethiopia. **20**, 2 (2007).
8. A. M. Bhutto, F. R. Soomro and K. Katakura, Leishmaniasis in Sindh, Pakistan: Outbreak and Review of Literature, *J. Pak. Assoc. Dermatol.,* **18**, 212 (2008).
9. S. Brooker, N. Mohammed, K. Adil, S. Agha, R. Reithinger, M. Rowland, I. Ali, J. Kolaczinski, Leishmaniasis in Refugee and Local Pakistani Populations, *Emerg. Infect. Dis.*, **10**, 1681 (2004).
10. J. L. Mclaughlin, L. L. Rogers, J. E. Anderson, The Use of Biological Assays to Evaluate Botanicals, *Drug Infor. J.,* **32**, 513 (1998).
11. N. Ghorai, S. Chakraborty, S. Gucchait, S. K. Saha and S. Biswas, Estimation of Total Terpenoids Concentration in Plant Tissues Using a Monoterpene, Linalool as Standard Reagent, *Protocol Exchange,* **2012,** 055 (2012).
12. E. L. C. de Amorim, A. J. C. Corrêa, de Melo JG, T. J. D. S. P. Sobrinho, V. T. Nd. Ad. Castro, Standard Operating Procedures (SOP) for the Spectrophotometric Determination of Phenolic Compounds Contained in Plant Samples, INTECH Open Access Publisher; (2012).
13. Y. S. Park, S. T. Jung, S. G. Kang, B. G. Heo, P. Arancibia-Avila, F. Toledo, J. Drzewiecki, J. Namiesnik, S. Gorinstein, Antioxidants and Proteins in Ethylene-treated Kiwifruits, *Food Chem.,* **107,** 640 (2008).
14. D. O. Kim, S. W. Jeong and C. Y. Lee, Antioxidant Capacity of Phenolic Phytochemicals from Various Cultivars of Plums. *Food Chem.*, **81**, 321 (2003).
15. M. You, D.B. Wickramaratne, G. L. Silva, H. Chai, T. E. Chagwedera, N. R. Farnsworth, G.A Cordell, A. D. Kinghorn and J. M. Pezzuto (-)-Roemerine, an Aporphine Alkaloid from *Annona senegalensis* that Reverses the Multidrug-Resistance Phenotype with Cultured Cells. *J. Nat. Prod.,* **58**, 598 (1995).
16. T. Geetha and N. Geetha, Phytochemical Screening, Quantitative Analysis of Primary and Secondary Metabolites of *Cymbopogan citratus* (DC) stapf. Leaves from Kodaikanal hills, Tamilnadu, *Int. J. Pharm.Tech. Res.*, **6**, 521 (2014).
17. S. Zaib and M. R. Khan, Protective Effect of *Cucurbita pepo* Fruit Peel against CCl_4 Induced Neurotoxicity in Rat, *Pak. J. Pharmaceu. Sci.,* **27**, 1967 (2014).
18. X. P. Nunes, F. S. Silva, J. R. Gd. S. Almeida, F. J. M. Barbosa, J. T. de Lima, R. L. A. de Araújo, L. J. Q. Júnio, Biological Oxidations and Antioxidant Activity of Natural Products, INTECH Open Access Publisher, (2012).
19. K. C. Huang, *The pharmacology of Chinese Herbs*, CRC press, (1998).
20. N. Sultana, A. Ata, Oleanolic Acid and Related Derivatives as Medicinally Important Compounds, *J. Enzyme Inhib. Med. Chem.*, **23**, 739 (2008).
21. B. A. Shah, G. N. Qazi and S. C. Taneja, Boswellic Acids: A Group of Medicinally Important Compounds, *Nat. Prod. Rep.,* **26**, 72 (2009).
22. M. N. Laszczyk, Pentacyclic Triterpenes of the Lupane, Oleanane and Ursane Group as Tools in Cancer Therapy, *Planta Med.,* **75**, 1549 (2009).
23. A. Petronelli, G. Pannitteri, U. Testa, Triterpenoids as New Promising Anticancer Drugs. *Anti-cancer Drugs*, **20**, 880 (2009).
24. G. Di Carlo, N. Mascolo, A. A. Izzo, F. Capasso, Flavonoids, Old and New Aspects of a Class of Natural Therapeutic Drugs, *Life sci.,* **65**, 337 (1999).
25. P. Montoro, A. Braca, C. Pizza, De N. Tommasi, Structure-Antioxidant Activity Relationships of Flavonoids Isolated From Different Plant Species, *Food Chem.,* **92**, 349 (2005).
26. M. P. Kähkönen, A. I. Hopia, H. J. Vuorela, J. P. Rauha, K. Pihlaja, T. S. Kujala, M. Heinonen, Antioxidant Activity of Plant Extracts Containing Phenolic Compounds, *J. Agr. Food Chem*., **47**, 3954 (1999).
27. M. Naczk, F. Shahidi, Extraction and Analysis of Phenolics in Food, *J. Chromatogr.*, **1054**, 95 (2004).
28. Z. Ladan, J. Amupitan, O. Oyewale, R. Ayo, E. Temple, E. Ladan, Phytochemical Screening of the Leaf Extracts of *Hyptis spicigera* Plant, *Afr. J. Pure Appl. Chem*., **8**, 83 (2014).
29. B. A. Wani, D. Ramamoorthy, B. A. Ganai, Preliminary Phytochemical Screening and Evaluation of Analgesic Activity of Methanolic Extract of Roots of *Gentiana kurroo* Royle in Experimental Animal Models, *Int. J. Pharm. Pharm. Sci*., **3**, 164 (2011).
30. A. A. Zahi, A. A. Rahuman, S. Pakrashi, D. Ghosh, A. Bagavan, C. Kamaraj, G. Elango, M. Chatterjee, Evaluation of Antileishmanial Activity of South Indian Medicinal Plants Against *Leishmania donovani*, *Exp. Parasitol.*, **132**, 8 (2012).
31. D. O. Santos, C. E. Coutinho, M. F. Madeira, C. G. Bottino, R. T. Vieira, S. B. Nascimento, A. Bernardino, S. C. Bourguignon, S. Corte-Real and R. T. Pinho, Leishmaniasis Treatment - A Challenge That Remains: a review, *Parasitol. Res.,***103**, 1 (2008).