Larvicidal, insecticidal, brine shrimp cytotoxicity and anti-oxidant activities of *Diospyros kaki* (L.) reported from Pakistan

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Abstract: *Diospyros kaki* is cultivated in different agro-ecological zones of Pakistan, especially in Malakand division. The current study was designed to investigate the hide potential of the vulnerable species of the plant. Aqueous extracts of *Diospyros kaki* leaves were screened for larvicidal, insecticidal cytotoxic and antioxidant activities. The extract exhibited moderate to outstanding larvicidal activity (100 to 28%) at 100, 80, 70, 50, 40, 30, 20 and 10% concentrations respectively after 24 hours, showing 42% LC_{50}. Permitrin displayed 100% lethality at 0.3%. The extract demonstrated outstanding cytotoxic action against brain shrimps nauplii (Artemia salina), showing 10 ppm LC_{50} which is closed to the LC_{50} (9.8µg/ml) of standard drug Etoposide. Similarly profound insecticidal potential (100%) was recorded after 15 min against *Cimex lectularius*. In DPPH scavenging activity the extract demonstrated moderate 30.22%, while Quercetin, Gallic acid and Acetic acid showed 98, 96 and 97% activity respectively at 100ppm. Thus on the basis of our finding it could be concluded that the decoction of the leaves of *D. kaki* is a good natural alternative for the control of insects and neoplasia.

Keywords: Pakistan, *Diospyros kaki*, water crude extract, larvicidal, insecticidal, cytotoxic and anti-oxidant activities.

INTRODUCTION

Since the beginning of civilization, people from all over the world used plants as a source of medicine. Evidence shows that human being used plants to the treatment of different diseases, such as Chinese Emperor in 2800 BC, Babylon in 1770 BC and Ancient Egypt in 1550 BC (Shinwari and Qaisar, 2011). Medicinal plants are one of the best sources of income in developing countries. Most of the Medicinal plants are belonging to flowering plants. More than 10% out of 32000 species of higher plants are used medicinally (Prance, 2001). Numerous strategies have been developed to investigate biological compounds from various resources like animals, plants and micro-organism. Traditional medicines are included in such resources. Plants are the rich source for the discovery of novel and potent compounds (Nitta *et al*., 2002). The medicinal values of plants are due to the presence of secondary metabolites (Parekh and Chanda, 2007). The discovery of plants having medicinal value is important equally for medicinal and agriculture sectors all around the world, and allows the establishment of alternative ways for medicinal crop propagation which offers better social and economic benefits (Hamilton, 2004).

D. Kaki belonging to family Ebenaceae, genus *Diospyros* and contains hundreds of species (Duangjai *et al*., 2009). *D. kaki* is one of the medicinal plant native to Japan, China, Pakistan, India and Myanmar. It is fully naturalized in Algeria, Afghanistan, Australia, Egypt, Brazil, Indonesia France, Republic of Korea, Israel, Italy, Philippines, Palestine, Union of Soviet Socialist Republic (Former), Russian Federation, United States of America and is commonly known as Japanese persimmon (Singh and Joshi, 2011). *D. kaki* is locally known as "sur amlook", "Parsimmon", also cultivated for its edible fruits in Pakistan particularly in Dir, Swat and Malakand. Traditionally *D. kaki* is use as styptic and astringent. The fruits have diversified properties depending on its stage of ripeness. Generally the fruit is anti-tussive, laxative, astringent, stomachic and nutritive. The ripe fruit in raw form are used for the treatment of hemorrhoids and constipation, after cooking for diarrhea treatment, in dried and powdered form the fruit is used for bronchial complaints and dry cough respectively. Hypertension can also be treated when juice of unripe fruit is used. The fruits are considered to be anti-febrile, demulcent and antivininous (Singh and Joshi, 2011).

It has been reported that the tannin isolate from ripe fruit have very strong detoxifying activity against the venoms of two snake species i.e. *Trimeresums flavoviridis* and *Laticauda semifasciata* (Singh and Joshi, 2011). The fruit has active constituents which can inactivate bacteria toxin of *Straphylococcus alpha*, *Bordetella pertussis*, *Clostridium tetani* and *Diptheria* (Mallavadhani *et al*., 1998). The leaves of *D. Kaki* have antioxidant, anti-thrombotic activity (Chen *et al*., 2007), liver-protecting activity (Lim, 2012) , anti-allergy (Park, 2000) and anti-cancer activity (Jo *et al*., 2010), anti wrinkle effect of Polyphenols isolated from leaves (An *et al*., 2005) and are also used to treat hypertension (Funayama and Hikino,

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1979). The literature shows that astragalin and isoquercitrin have been isolated from the leaves of *D. kaki*, which are responsible for hypotensive effect (Funayama and Hikino, 1979). In our previous study we have reported the anti-microbial activity of *D. kaki bark* (Shah *et al*., 2012).

Based on the previous literature the present study was carried out to evaluate the larvicidal, insecticidal, brine shrimp cytotoxicity and antioxidant activity of the aqueous extract of *D. kaki* leaves.

MATERIAL AND METHODS

Plant material

The leaves (01 Kg) of *Diospyros kaki* (L) were collected from village Matta, district Swat, Khyber Pakhtunkhwa, Pakistan. The plant materials were authenticated by Professor Mehboob Rehman, Botany Department, Jahanzeb College, Swat, Pakistan. A voucher specimen was maintained at the Herbarium, Department of Botany, University of Malakand. The plant materials were surface sterilized with double distilled water.

The plant materials were thoroughly washed with double distilled water, dried at room temperature and chopped in to small pieces by mean of electric blender at Pharmacognosy lab, Department of Pharmacy, University of Malakand. The course powder (300 gm) was then socked in distilled water and heated genteelly with constant stirring. The aqueous extract was filtered through Whatman No.1 filter paper. The filtrates were concentrated at water bath at controlled temperature.

Larvicidal activity

The larvicidal activity of aqueous crude extract of *Diospyros kaki* against *Culex quinquefasciatus* was performed as per (Ilahi *et al*., 2012, Okumu *et al*., 2007). The larvae of *C. quinquefasciatus* were reared in vector control laboratory of the Department of Biotechnology, University of Malakand under standard environmental condition (Mathivanan *et al.*, 2010). Third and fourth instars larvae were collected in plastic bucket and identified by Prof. Dr. Suliman, Dean of Health Sciences, Department of Microbiology, Hazara University, Khyber Pakhtumkhwa, Pakistan. The larvicidal activity was carried out at different concentration of decoction. The larvae were then transferred to the test medium and control was maintained simultaneous in distilled water. Three sets of 10 glass beakers each of 100 ml were laden on a laboratory bench. Brewer's Yeast was supplied as a larval food in same concentration to each beaker. The standards were also maintained having Permethrin 0.03%. The larval mortality was recorded in test sample, control and standard medium. The larva was said to be dead when they failed to move by needle probing. The experiment was conducted under laboratory condition at 25-30^{o}C.

Insecticidal activity

Aqueous extract of *D. kaki* leaves was applied to check the insecticidal activity. Total 80 x 3 adult Bed-Bugs (*Cimex lectularius*) were collected from different sites of Thana, Khyber Pakhtunkhwa, Pakistan. A total of 240 Petri-plates were surface sterilized with double distilled water. In the sterilized plates 20 Bed-Bugs were exposed to different concentration of aqueous crude extract (80, 75 and 50%). A control was laden with out any plant extract. The effect of *D. kaki* extract was determined after 24 h by counting the number of dead insects. When the insects failed to move after probing with a sharp needle in cervical region were recorded as dead. The experiment was conducted in triplicate subsequently the percentage of insecticidal mortality was determined.

Brine shrimp cytotoxicity

In the experiment the material and reagents used were; sea salt (40g/L of distilled water having pH 7.4), test sample, tray for hatching eggs, micro pipettes, lamp for attraction of larvae, distilled water and methanol. The procedure of Mayer *et al.* (2007) brine shrimps cytotoxicity was followed with slight modifications.

The test sample (25mg) was dissolved in 2.5ml of water to prepare a stock solution. In separate vials three concentrations 1000, 100 and 10ppm were prepared. Ten shrimp's nauplii were transferred to each vial and adjust the final volume up to 10 ml with sea salt water. The experiment was performed in triplicate and incubates at 25±1^{o}C for 24h. Afterwards, the numbers of dead shrimp's nauplii were counted and the percentage was calculated.

Anti-oxidant activity

The Anti-oxidant activity of different concentration (100, 80, 60, 40 and 20µg/ml) of *D. kaki* aqueous extract, quercetin, Gallic acid and ascorbic acid was determined by using 2, 2-diphenyl-1-picryl-hydrazyl (DPPH, free radical scavenger) (Braca *et al*., 2002). A control was prepared by mixing 1 ml of DPPH in 5 ml of methanol. Then 5 ml of each concentration of the extract was mixed with 0.002% of DPPH solution and incubated for 30 min in dark at room temperature. The absorbance was measured at 517 nm using UV-spectrophotometer. To calculate the scavenging capability of DPPH radical the following formula was used.

$$\text{Scavenging activity (\%)} = \frac{\text{Control - Sample}}{\text{Control}} \times 100$$

Where Control is the absorbance of Methanol + DPHH and Sample is the absorbance of the extract. The experiment was performed in triplicate.

RESULTS

Larvicidal activity

The aqueous extract of *D. kaki* exhibited marked activity against the larvae of *C. quinquefasciatus* mosquito. The

phenotypic change caused by the plant extract with respect to time is summarized in table 1. The colony establishment and their distribution were concentration depended. In first 3h with 80% decoction, colony established but distributed in the next 09h with 15% mortality while in the group of Permethrin (0.03%) colony distributed with 100% mortality within 6 h. In the control group no larvicidal activity was observed in 24 h (table 1). The extract demonstrated a dose dependent larvicidal activity showing 40.2% LC_{50}. 100 and 50% decoction caused 100 and 60% mortality respectively within 24 h (table 2).

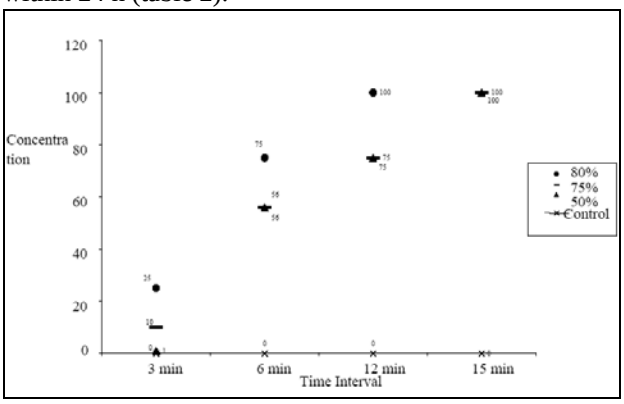

Fig. 1: Mortality index of water crude extract of *Diospyros kaki* leaves on Bed-Bug.
Standard: Permethrine; Control: Distilled water

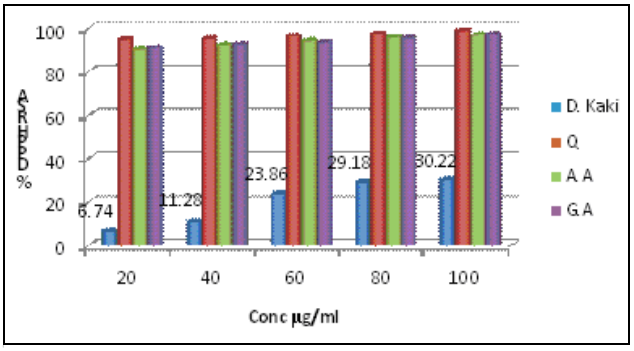

Fig. 2: DPPH radical scavenging effect of *D. kaki* extract.
Each percent point represents mean ± SEM values of three independent observations.

Insecticidal activity

The extract displayed a time and concentration dependent insecticidal activity against the common Bed-Bugs. The highest concentration (80%) was comparatively more potent and caused 100% mortality after 12 min of the exposure to the extract. Moreover all the tested concentration showed 100% mortality within 15 minutes (table 4).

Brine shrimp cytotoxicity

The Brine shrimp cytotoxicity results are summarized in table 3. The plant extract demonstrate a concentration dependent cytotoxic activity with 10ppm ED_{50} value. The extract caused 100% mortality at a concentration of 100 ppm. The reference standard drug (Etoposide) showed 9.8 µg/ml ED_{50}. The toxicological effect of the plant extract is comparable to the effect of the standard drug (Etoposide). The results hence indicate that the plant extract has highly potent cytotoxic constituents.

Antioxidant activity

The extract showed IC_{50} value above 100 ppm which indicates weak DPPH radical scavenging potential of the aqueous extract. Maximum antioxidant (30.22%) was observed at highest concentration (100ppm). While the reference standards, quercetin, gallic acid and ascorbic acid displayed outstanding antioxidant activity, 98.87, 96.87 and 97.21 respectively.

DISCUSSION

Mosquito is a group of organisms which act as a vector for transmitting diseases such as dengue fever, yellow fever, malaria etc (Morse, 1995, Gubler, 2002). To reduce the density of mosquito it is necessary to kill the larvae before they reach to adult stage. In the current study the aqueous extract revealed marked larvicidal activity (40.2% LC_{50}). The highest concentration 100% decoction) killed the entire larva within 24h (table 2). Different insecticides in the market are available to kill mosquitoes but they produces a large number of problems, such as environmental pollution and development of resistant against the insecticides (Kunz and Kemp, 1994, Biondi *et al*., 2012). Natural insecticides obtained from plants are bioactive compounds and have no or little harmful effect on environment and are the good alternatives of synthetic insecticides (Consoli *et al*., 1998, Zabel *et al*., 2002, Senthil Nathan, 2007). A plethora of research is available, emphasizing the significance of plants extract as efficient insecticides. It is well documented that the extract obtained from the leaves of *Azadirachta indica* (neem) has shown 75% mortality against Anopheles mosquitoes (Okumu *et al*., 2007). The aqueous extract of *Diospyros kaki* showed strong insecticidal against Bed-Bug and caused 100% mortality after 15 min of the exposure to the extract. The extracts of *D. kaki* may be a best natural insecticide and an economical and safe alternative of synthetic insecticides.

Brine shrimps lethality assay is a preliminary study for the confirmation of anticancer activity (Anderson *et al*., 1988). Cancer is one of the challenging diseases of the current era for the researchers. Numerous scientists are trying for the discovery and development of effective and safe remedies from natural sources for the treatment of cancer (Cherny and Catane, 2003). The present study is an attempt to uncover the hidden potential of *D. kaki* for the treatment of cancer. The brine shrimp cytotoxic assay revealed that the plant extract possesses strong toxicological effect (10ppm LC_{50}) and further investigation may leads to the isolation of active principle responsible for cytotoxic activity and may uncover the cure of cancer.

Table 1: Concentration dependent Phenotypic change in larval colonies after 100, 80 and 50% leaf crude extract of *Diospyros kaki*

Concentration of Extract/Drug	3 h	6 h	12 h	24 h
100%	Colony established	Colony disturbed with no mortality	20 ± 1.45% mortality	100±0.00% mortality
80%	Colony established	Colony disturbed with no mortality	15 ± 1.17% mortality	75±1.45% mortality
50%	Colony established	Colony established	Colony disturbed with no mortality	30.66±1.85% mortality
Permethrin	Colony disturbed with 29.66±1.20% mortality	100±0.00% mortality	100 ± 0.00% mortality	100±0.00% mortality
Control	Colony established	Colony established	Colony established	Colony established

Table 2: Larval mortality at 100, 80, 70, 50, 40, 30, 20 and 10% leaves crude extract of *Diospyros kaki* after 24 h

Concentration (%)	No of dead larva	% mortality	LC_{50}
100	20±0.00	100	40.2%
80	15.67±0.33	75.00	
70	13.67±0.33	68.30	
50	12±0.58	60.00	
40	9.33±0.33	46.65	
30	6±0.33	33.35	
20	5±0.67	28.35	
10	1.67±0.33	08.35	
Standard drug (permthrine)	20.0±0.00	100	
Control	--	--	

Values are expressed as means of three replicated ± SEM, n=20.

Table 3: Concentration and Time dependent Percent mortality of aqueous extract of *D. kaki* against Bugs

Concentration effect	3 min	6 min	12 min	15 min	18 min
80% crude extract	25.33±0.882%	75.33±2.029%	100.0±0.00%	------	--------
75% crude extract	10.66±1.203%	56±2.312%	75±1.734%	100.0±0.00%	--------
50% crude extract	5.66±1.203 %	55.66 ±1.203%	75±1.454%	100.0±0.00%	--------
Control	0.0±0.0%	0.0±0.0%	0.0±0.0%	0.0±0.0%	0.0 ± 0.0%

Each percent value represents mean ± SEM of three independent replicate

Table 3: Concentration dependent cytotoxic potential of crude methanolic extracts of *D. kaki* bark against Brine shrimps nauplii

Treatment	Conc. (ppm)	Total No's of shrimps tested	Total No's of shrimps killed	% mortality	LC_{50} (ppm)
Test sample	10	20	10.0±0.88	50	10
	100	20	20.0±0.00	100	
	1000	20	20.0±0.00	100	

Conc: Concentration. Values are expressed as the mean ± SEM of three independent observations. Standard drug; Etoposide LD_{50} = 9.8 µg/ml.

The in vitro antioxidant activity against free radical DPPH revealed that the aqueous extract of *D. kaki* could not significantly (30.22%) affected the DPPH radical scavenging effect (fig. 2).

Thus on the basis of our findings it is concluded that highly potent constituents are residing in the aqueous extract of *D. kaki* leaves. The extract exhibits strong lavicidal, insecticidal, cytotoxic and moderate antioxidant potentials. Therefore the plant extract is recommended for further investigation through advance techniques used for natural product isolation and structure elucidation of the bio-active compound, such efforts may result in the discovery of novel compounds possessing a wide range of bioactivity.

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