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Phytochemical analysis, anticancer and antioxidant studies of TRIVENI plants: *In-vitro* studies

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Abstract

The TRIVENI include *Azadirachta indica* (neem), *Ficus religiosa* (peepal), and *Ficus benghalensis* (bohar) are recognized for their rich cultural significance and therapeutic benefits in traditional Indian medicine. In the present study, the extracts from the TRIVENI plants were prepared for phytochemical, cytotoxic effects (MIAPACA pancreatic cancer cell line and Fr2 breast epithelial cell line) and antioxidant potential (DPPH) studies. The results from the present study suggested that the TRIVENI plants possess phenols and flavonoids contents. The TRIVENI extracts showed anticancer potential against MIAPACA pancreatic cancer cell line and safe on Fr2 breast epithelial cell line. The free radical induced oxidative stress can cause gene mutations to increase transcription factors for cancer so antioxidants can be effective to reduce cancer progress. Interestingly, in the present study, extracts also showed antioxidant properties by sequencing free radicals, which indicates its potential against cancer. Therefore, further *in-vivo* studies on antioxidant and anticancer potential of TRIVENI plant would be beneficial to explore the mechanism of action.

Keywords: Pancreatic cancer, TRIVENI, *Azadirachta indica*, *Ficus religiosa*, and *Ficus benghalensis*, MIAPACA, DPPH

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Introduction

In pancreatic cancer malignant cells initiates to grow in the pancreas tissues, which produces digestive hormones and juices to regulate blood sugar. Exocrine pancreas cells release the digestive juices whereas pancreatic endocrine cells synthesise the hormones (Li *et al.* 2004). The pancreatic cancers majority start in the exocrine cells. Albumin-bound paclitaxel, gemcitabine, 5-FU (fluorouracil) and irinotecan are clinically used for the treatment of pancreatic cancer, however they are associated with the side effects such as hair loss, fatigue, loss of appetite, abdomen pain in addition with drug resistance is another problem related with failure of these drugs (Conroy *et al.* 2011; Halperin and Varadhachary 2014). Hence, medicinal plants base treatment are the alternative and safe options reduced chemotherapy related problems and resistance (Kumar *et al.* 2011). India has one of the oldest, richest and most diverse cultural traditions associated with the use of medicinal plants in the form of folk medicine. The plant drugs have received considerable preference because of their presumed safety and potential nutritional and therapeutic effects (Hamed 2011). Several herbs used in Indian medicine system "Ayurveda". Plants and other natural products are the power house of therapeutic constituents such as lutein, rutin, β-carotene, gallic acid, stigmasterol, kampeferol, apigenin, ellagic acid, quercetin, linalool, terpinolene, ascorbic acid, myricetin, ferulic acid, naringenin, luteolin, ortho-coumaric acid, para-coumaric acid, etc., which possess therapeutic efficacy that make them as complimentary medicine helpful in the management of multiple disorders with negligible side effects (Kaur and Kumar, 2012; Kateel *et al.* 2014). Additionally, therapeutically active constituents act by targeting multiple mechanisms and produce synergic action (Aslam *et al.* 1978; Subramanian *et al.* 1978; Chandrasekar *et al.*, 2010). 'TRIVENI' is the combination of three Miracle plants namely neem (*Azadirachta indica*), peepal (*Ficus religiosa*) and bohar (*Ficus benghalensis*) grow together in small distance. TRIVENI plants have a lot of ecological and mythological significance (Hamed, 2011; Saxena *et al.* 2012; Kumar *et al.* 2013). These plants are the powerhouse of such therapeutic active constituents, which possess tremendous pharmacological activity. Therefore, the present study was designed to evaluate the phytochemical analysis and anti-pancreatic activity of extracts of TRIVENI plants.

Materials and methods

Collection and authentication of plant material

Leaves *Azadirachta indica A. Juss.* (Meliaceae), *Ficus religiosa* (Moraceae) and *Ficus benghalensis* (Moraceae) were collected from Chaudhary Bansi Lal University Bhiwani (Haryana). The plant was authenticated from Raw Material Herbarium and Museum, CSIR-National Institute of Science Communication and Information Resources, New Delhi, vide reference no, NISCAIR/RHMD/ Consult/2018/3273-74-1, NISCAIR/RHMD/Consult/2018/3273-74-2 and NISCAIR/RHMD/Consult/2018/3 273-74-3 respectively. Leaves of these plants were used for the present study.

Preparation of extracts with various solvents

Leaves *Azadirachta indica A. Juss.* (Meliaceae), *Ficus religiosa* (Moraceae) and *Ficus benghalensis* (Moraceae) were collected and washed and cut into fine pieces. Collected leaves were dried at room temperature (Sowmya and Nanjammanni, 2017). The dried leaves were grind to coarse powder using a grinder (Saikiaand Bora, 2018). 110g coarse of dried leaves of *Azadirachta indica*, *Ficus religiosa* and *Ficus benghalensis* treated with petroleum ether to remove the fatty acid present in the samples. Then dried the samples and extracted with different ratios of 99% ethanol, ethyl acetate and aqueous extracts 48 hours at room temperature using Soxhlet apparatus. The solvents was evaporated from the samples at 65°C by water bath (Vijayanand and Sanjana, 2017).

Preliminary phytochemical analysis

The phytochemical analysis in the TRIVENI plants extracts were done as per the method given by Ahmed *et al.*, 2013 and Hasan *et al.*, 2016.

Determination of total flavonoid and phenol content

The total phenol and flavonoid content were measured using method given by Kujala *et al.*, 2000; Siddhuraju *et al.*, 2007.

In vitro Cytotoxicity and anticancer activity

Fr2 (Breast epithelial healthy cell lines) and human cancer cell lines MIAPACA (Pancreatic) were procured from U.S. National Cancer Institute (NCI). MIAPACA cell line were grown in Dulbecco's minimal essential medium supplemented with 10 % FBS, 2-mM L-glutamine, 3-mM sodium pyruvate, 100- units/mL penicillin, 100-µg/mL streptomycin in a carbon dioxide incubator (New Brunswick, Galaxy 170R, Eppendorf) at 37°C, 5% CO2 and 98% RH. Cell viability was determined by thiazolyl blue tetrazolium bromide (MTT) and Trypan blue assays. Briefly, 7 × 103 cells were seeded into 96- well microtitre plate and differentiated for 48h.After 48h treatment with different concentrations, MTT dye (2.5 mg/mL) was added and incubated for 4 h at 37 °C; after incubation, the media was aspirated and 150µL/well DMSO was added and read at 570nM on Mutliskan GO plate reader (Thermo Fisher Scientific, Waltham, MA, USA). Molecules were screened against two cell lines MIAPACA (liver) Fr2 (Breast epithelial cell lines). IC50 of compounds which shows % inhibition at 10µM was determined by using Prism software.

In-vitro antioxidant method using 1, 1-Diphenyl, 2-picryl-hydrazyl (DPPH) free radical scavenging assay

DPPH was used to study free radical scavenging activity (in tripilcate) of the ethyl acetate extracts of *Azadirachta indica* (Neem), *Ficus Religiosa* (Peepal) and *Ficus benghalensis* (Banyan Tree) as per the method given by Sathya *et al.*, 2012.

Results

Preliminary phytochemical analysis of extracts of TRIVENI plants

Preliminary phytochemical analysis of different extracts of *Azadirachta indica, Ficus religiosa* and *ficus benghalensis* showed the presence or absence of various phytochemicals. The results of phytochemical analysis of selected extracts have been shown in Table 1, 2 and 3.

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Table 1: Preliminary phytochemical analysis of extracts of *Azadirachta indica* leaves

Test	Ethyl acetate extract of *Azadirachta indica*	Ethanol extract of *Azadirachta indica*	Aqueous extract of *Azadirachta indica*
Carbohydrates			
Fehling's test	+	+	+
Alkaloids			
Dragondroff's test	+	+	-
Coumarin glycosides			
Fluorescence test	+	+	-
Saponins			
Foam test	-	-	+
Flavonoids			
Shinoda test	+	+	-
Phenols			
Ferric chloride test	+	+	-
Steroids and terpenoids			
Salkovaski Test	+	+	-

+ denotes present and – denotes absent

Table 2: Preliminary phytochemical analysis of extracts of *Ficus Religiosa* Leaves

Test	Ethyl acetate extract of *Ficus Religiosa*	Ethanol extract of *Ficus Religiosa*	Aqueous extract of *Ficus Religiosa*
Carbohydrates			
Fehling's test	+	+	+
Alkaloids			
Dragondroff's test	+	+	-
Coumarin glycosides			

Fluorescence test	+	+	-
Saponins			
Foam test	-	-	+
Flavonoids			
Shinoda test	+	+	-
Phenols			
Ferric chloride test	+	+	-
Steroids and terpenoids			
Salkovaski Test	+	+	-

+ denotes present and – denotes absent

Table 3: Preliminary phytochemical analysis of extracts of *Ficus benghalensis* Leaves

Test	Ethyl acetate extract of *Ficus benghalensis*	Ethanol extract of *Ficus benghalensis*	Aqueous extract of *Ficus benghalensis*
Carbohydrates			
Fehling's test	+	+	+
Alkaloids			
Dragondroff's test	+	+	-
Coumarin glycosides			
Fluorescence test	+	+	-
Saponins			
Foam test	-	-	+
Flavonoids			
Shinoda test	+	+	-

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Phenols			
Ferric chloride test	+	+	-
Steroids and terpenoids			
Salkovaski Test	+	+	-

+ denotes present and – denotes absent

Determination of total flavonoid content

The total flavonoid content in ethyl acetate, ethanol and aqueous extracts were estimated using quercetin as standard. The quercetin solutions of efficient (R^2) = 0.998. The equation of standard curve is y = 2.100x -0.196. Total flavonoid content was found to be 0.86±0.04 mg of quercetin equivalents/g of dry weight. Total flavonoid content in ethyl acetate, ethanol and aqueous extracts of Leaves *Azadirachta indica A. Juss.* (Meliaceae), *Ficus religiosa* (Moraceae) and *Ficus benghalensis* (Moraceae) is shown in Table 4, 5 and 6.

Table 4: Total flavonoid content in *Azadirachta indica extracts*

Extract	Total flavonoid content (mg of quercetin equivalents/g)
Ethyl acetate extract	0.19±0.06
Ethanol	0.82±0.02
Aqueous	0.04±0.01

Values are expressed as Mean±S.E.M.; n=3

Table 5: Total flavonoid content in *Ficus religiosa extracts*

Extract	Total flavonoid content (mg of quercetin equivalents/g)
Ethyl acetate extract	66±0.12
Ethanol	49±0.05
Aqueous	Absent

Values are expressed as Mean±S.E.M.; n=3

Table 6: Total flavonoid content in *Ficus benghalensis extracts*

Extract	Total flavonoid content (mg of quercetin equivalents/g)
Ethyl acetate extract	26±0.19
Ethanol	31±0.03
Aqueous	Absent

Values are expressed as Mean±S.E.M.; n=3

Determination of total phenolic content

Gallic acid was used as standard. The gallic acid solutions of different concentration (20-100 ppm) confirmed to Beer's Law at 725 nm with regression co-efficient (R^2) = 0.998. The equation of standard curve is y = 0.011x + 0.085. Total phenolic content in various extracts is shown in Table 7, 8 and 9.

Table 7: Total phenolic content in *Azadirachta indica* leaves extract

Extract	Total phenolic content (mg of gallic acid equivalents/g)
Ethyl acetate extract	80.76±0.31
Ethanol	80±0.12
Aqueous	18.05±0.03

Values are expressed as Mean±S.E.M.; n=3

Table 8: Total phenolic content in *Ficus religiosa* leaves extract

Extract	Total phenolic content (mg of gallic acid equivalents/g)
Ethyl acetate extract	68.14±0.02
Ethanol	40.47±0.06
Aqueous	08.23±0.03

Values are expressed as Mean±S.E.M.; n=3

Table 9: Total phenolic content in *Ficus benghalensis* leaves extract

Extract	Total phenolic content (mg of gallic acid equivalents/g)
Ethyl acetate extract	29.14±0.02
Ethanol	3.47±0.06
Aqueous	0.23±0.03

Values are expressed as Mean±S.E.M.; n=3

Cytotoxicity study antipancreatic cancer activity of extracts of ethyl acetate *Azadirachta indica, Ficus religiosa* and *Ficus benghalensis* on Fr2 and MIAPACA cell lines

Cytotoxicity refers to the ability of certain chemicals or cell mediators to destroy living cells. IC50 value is used to calculate the cytotoxicity of the drugs. IC_{50} is the concentration of extracts where the 50 % of cells die. Fr2 and MIAPACA cell line was used for test cytotoxicity profile of the extracts on normal cell and pancreatic cancer cell lines respectively. IC_{50} of the ethyl acetate *Azadirachta indica, Ficus religiosa and ficus benghalensis* on Fr2 and MIAPACA cell lines has been showed in Table 10.

Table 10: Cytotoxicity and anticancer activity of extracts of ethyl acetate *Azadirachta indica, Ficus religiosa and Ficus benghalensis*

Test compound	IC_{50} (µg/ml)	
	Fr2 (Normal cell line)	MIAPACA (Pancreatic cancer cell lines)
Azadirachta indica (20mg)	55.5	6.20
Ficus religiosa (20mg)	54	15.00
Ficus benghalensis (20mg)	25.5	17.09

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DPPH scavenging activity of ethyl acetate *Azadirachta indica, Ficus religiosa, Ficus benghalensis* and quercetin

The DPPH free radical scavenging activity of extracts was detected and compared with the quercetin. The ethyl acetate *Azadirachta indica, Ficus religiosa, Ficus benghalensis* and quercetin showed to scavenge the free radicals as indicated by increase in percentage inhibition, which represents antioxidant property of the extracts (Table 11).

Table 11: Percentage scavenging activity of ethyl acetate extract

S. No.	Conc. (µg/ml)	Quercetin	*Azadirachta indica*	*Ficus religiosa*	*Ficus benghalensis*
1.	20	44.33±0.62	86±0.61	89.03±0.11	79.33±0.21
2.	40	34.33±0.94	78.12±0.52	72.32±1.54	68.02±2.22
3.	60	23.36±0.31	68.28±0.26	69.23±0.43	62.18±2.6
4.	80	11.71±0.74	47.15±2.47	62.35±0.24	41.75±0.43
5.	100	2.06±0.49	33.19±0.21	51.12±0.28	34.29±0.23

Values expressed as % scavenging activity (mean ± SEM); n=3,

Discussion and Conclusion

Preliminary phytochemical analysis of ethanol, ethyl acetate extract and aqueous extracts of neem (*Azadirachta indica*), pipal (*Ficus religiosa*) and banyan tree (*Ficus benghalensis*) was carried out. The ethanol and ethyl acetate extracts of TRIVENI plants showed the presence of alkaloids, glycosides, phenols, flavonoids and terpenoids. Total phenolic and flavonoid content in ethyl acetate extracts was found to be higher than other extracts. Based on the preliminary phytochemical screening and total phenolic and flavonoid content ethyl acetate extracts of TRIVENI plants were studied for their cytotoxicity and anti-pancreatic cancer against human cell line. Cytotoxicity is one of the most important indicators for biological evaluation of drugs via different mechanism such as destruction of cell membranes, prevention of protein synthesis, irreversible binding to receptors etc. Cytotoxicity and anti-cancer activity

of *Azadirachta Indica, Ficus Religiosa* and *Ficus Benghalensis* extract was tested on human normal breast epithelial cell line (Fr2) and pancreatic cancer cell line (MIAPACA) using MTT assay. MIAPACA was derived from the pancreas adenocarcinoma. The cytotoxicity of test compounds was showed by growth inhibitory concentration (IC50) in which drug shows 50% cytotoxic effect against cancer cells. *Azadirachta Indica, Ficus Religiosa* and *Ficus Benghalensis* extract were studied on normal breast epithelial cell line (Fr2) for the selection of optimum and effective concentration of the drug for the further preclinical studies in future. It has been found that IC50 value of *Azadirachta Indica, Ficus Religiosa* and *Ficus Benghalensis on* human normal breast epithelial cell line (Fr2) were 55.5µg/ml, 54µg/ml and 25.5µg/ml respectively, which showed that *Azadirachta Indica* is safer than *Ficus Religiosa* and *Ficus Benghalensis* on healthy cell line. Furthermore, IC50 value of *Azadirachta Indica, Ficus Religiosa* and *Ficus Benghalensis* on pancreatic cancer cell line (MIAPACA) was 6.20µg/ml, 15µg/ml and 17.09µg/ml respectively, which showed that *Azadirachta Indica* has shown more anti-pancreatic cancer effect than *Ficus Religiosa* and *Ficus Benghalensis*. Pharmacologically, flavonoids and polyphenols inhibit the expression of NFkB, which is needed for cancer cell survival, angiogenesis and proliferation. In the present study, ethyl acetate extracts of *Azadirachta Indica, Ficus Religiosa* and *Ficus Benghalensis* has also shown the presence of flavonoids and polyphenols.

Furthermore, free radicals or other reactive oxygen species produced during aerobic metabolism in the body. Oxidative stress is also associated with the cancer. The human body induces oxidative stress from exogenous origins (e.g., ultraviolet rays) and endogenous origins (from mitochondria). When free radical induced oxidative stress crosses the capacity of the antioxidant system then gene mutations can lead to affect transcription factors for cancer. It has also been reported that anticancer agents and radiation therapy showed their positive effects by cell death of cancer cells (Kimura *et al.*, 1998; Mates *et al.*, 1999). Therefore, it will be worthful to investigate antioxidant profile of the plant which is potential as anticancer for increasing their effectiveness. Therefore, we have selected ethyl acetate extracts of *Azadirachta indica* (Neem), *Ficus Religiosa* (Peepal) and *Ficus benghalensis* (Banyan Tree)

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for *In vitro* antioxidant activity using 1, 1-Diphenyl, 2-picryl-hydrazyl (DPPH) free radical scavenging assay. Ethyl acetate extracts of *Azadirachta indica* (Neem), *Ficus Religiosa* (Peepal) and *Ficus benghalensis* (Banyan Tree) have shown remarkable scavenging efficiency on DPPH, demonstrating a tendency to scavenge the free radicals as indicated by increase in percentage inhibition, which represents their antioxidant potential. Overall, there are some reports available in literature suggest that the agents working to reduce oxidative pathways have been effective in the treatment of cancer. Figure 1: Possible mechanism of action of flavonoids in the management of pancreatic cancer has been shown.

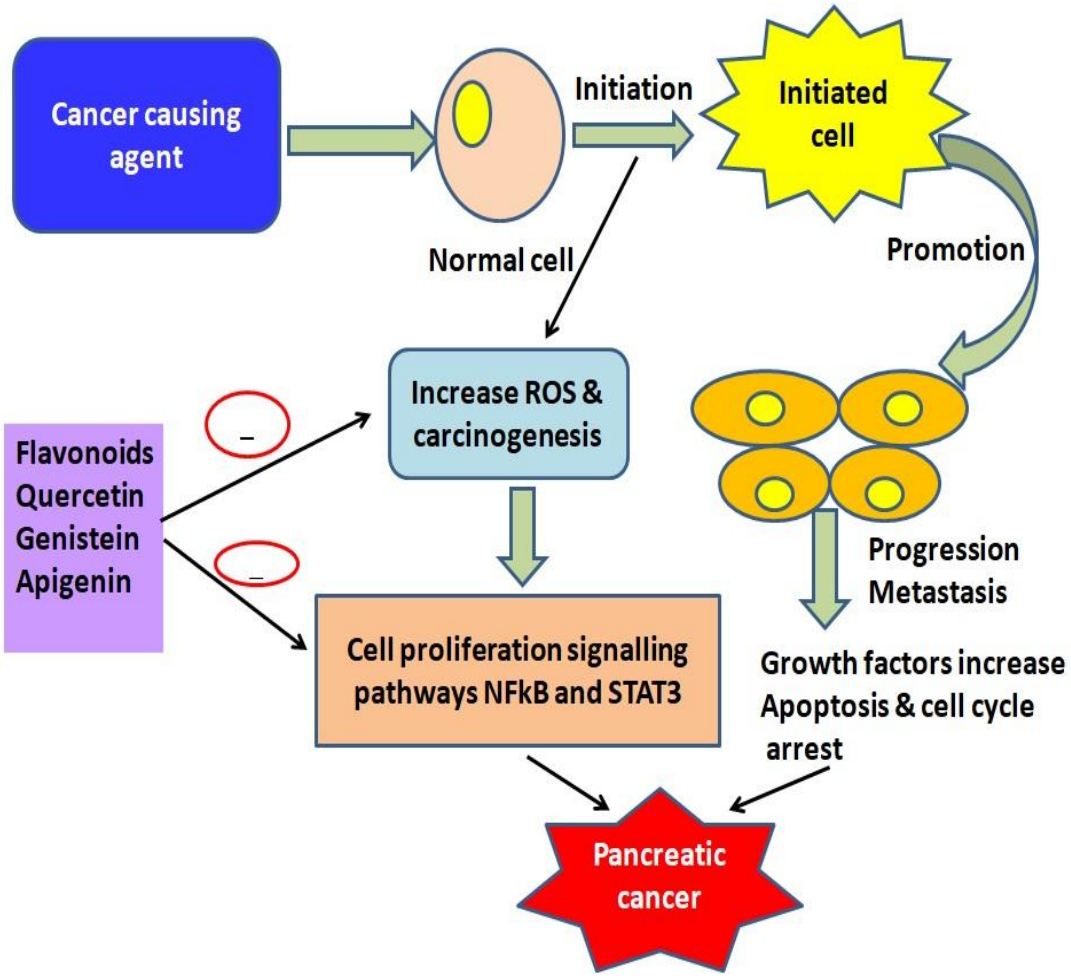

Figure 1: Possible mechanism of action of flavonoids in the treatment of pancreatic cancer

Conflict of interest statement

The authors have no relevant financial or non-financial interests to disclose.

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

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