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Antitumor effect of leaves of *Ravenala madagascariensis* Sonn., in PANC1 and SW1990 pancreatic cell lines

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Pancreatic cancer is the seventh leading cause of cancer-related deaths in developed countries with an average survival rate of less than 9%. Up to 80% of the patients with pancreatic cancer are found to be diabetic at the time of diagnosis. Leaves of *Ravenala madagascariensis* Sonn., have been traditionally used in the treatment of diabetes and was scientifically proven to be effective as an antidiabetic, hypolipidemic, renoprotective and antioxidant agent. In the present study, the antitumor effect of successive ethanolic leaf extract over two human pancreatic cancer cell lines PANC1 and SW1990 was evaluated by MTT assay. The shade dried, powdered leaves of *R. madagascariensis*, was subjected to successive soxhlet extraction with n-hexane, ethyl acetate followed by ethanol, concentrated and evaporated to dryness. The extract was subjected to preliminary phytochemical screening and was found to possess alkaloids, flavonoids, saponins, glycosides, phenols and tannins. The thin layer chromatography and high performance thin layer chromatography of various extracts of *R. madagascariensis*, was established. Based on the free radical scavenging potential, the ethanol extract was selected for further cytotoxicity studies. The ethanolic extract exhibited excellent cytotoxic effect against PANC1 and SW1990 with an IC_{50} value of 12.58 µg/mL and 18.9 µg/mL respectively. Thus the results validate the antitumor potential of *R. madagascariensis*, leaf extract against pancreatic cancer and further studies were aimed at the identification of active components responsible for the activity.

Keywords: *In-vitro* study, MTT assay, PANC1, Pancreatic cancer, *Ravenala madagascariensis*, SW1990.

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Introduction

Being ranked the seventh leading cause of death with a 5-year survival rate of 9%, pancreatic cancer is projected to be in third place in causing death surpassing breast cancer in future[1-3]. More than 45,750 pancreatic cancer-related deaths and 56,770 new cases have been reported in the United States in 2019[4]. Risk factors include smoking, family history, age, sex, diabetes mellitus, obesity, high-fat diet, infections due to *Helicobacter pylori* and periodontal diseases[5-9]. The high morbidity and lethality are due to its difficulty in diagnosis at early stages and poor prognosis[4]. Stronger cell invasion and metastasis to other tissues add on to the dreadful clinical manifestations of pancreatic cancer[10-12]. Pancreatic cancer is causally related to diabetes and insulin resistance. The insulin signalling cascade in skeletal muscles is impaired at multiple steps in pancreatic cancer. Up to 80% of patients with pancreatic cancer are found diabetic at the time of diagnosis[13]. Gemcitabine, the standard drug prescribed for pancreatic cancer has a poor response rate of less than 20%[14-16]. This inadequate response to the current therapeutics, late detection and drug resistance demands the need for extensive research in natural products.

Leaves of *Ravenala madagascariensis* Sonn., have been traditionally used in the treatment of diabetes[17]. The plant extract was scientifically proven to be effective as an antidiabetic, hypolipidemic, renoprotective and antioxidant agent[18-21]. The antioxidant potential of the plant provoked the investigation of its use in pancreatic cancer. The present study was aimed at exploring the antitumor effect of leaves of *R. madagascariensis*, in human pancreatic cell lines PANC1 and SW1990.

Materials and Methods

Chemicals, reagents and cell lines

All the chemicals and reagents were procured from certified suppliers and were of the highest analytical

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grade. PANC1 AND SW1990 cell lines were obtained from the National Centre for Cell Sciences, India, Pune (NCCS). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), penicillin (100 U/mL), and streptomycin (100 µg/mL) in a humidified atmosphere of 5% CO_2 at 37 °C.

Collection and authentication of plant material

Leaves of *R. madagascariensis*, (Strelitziaceae) were collected from Neyveli, Tamil Nadu during December 2018. Fully grown leaves were collected in fine dry weather and were stacked loosely in a vertical position in a shed for 3 weeks. The plant was identified and authenticated (No. PARC/2019/3958) by Plant Anatomy Research Centre, Chennai. The shade dried leaves were coarsely powdered and used for further studies. A voucher specimen has been deposited in the Department of Pharmacognosy, SRM College of Pharmacy, Kattankulathur, Tamil Nadu, India.

Extraction of plant material

Successive extraction was performed by Soxhelation of shade dried and coarsely powdered leaves of *R. madagascariensis*, with n-Hexane, Ethyl acetate, and ethanol. The successive extracts were then concentrated by using a rotary evaporator at 40 ºC and the dried extracts were weighed and stored at -4 ºC. The percentage yield was calculated using the formula,

$$\text{Percentage yield} = \frac{W_2 - W_1}{W_0} X100$$

Where W_2 is the weight of the extract and the container, W_1 the weight of the container alone and W_0 the weight of the powdered plant material subjected to extraction. Preliminary phytochemical screening was carried out to identify the presence of various phytochemical constituents in all the three successive extracts[22].

Thin layer chromatography (TLC) profiling

TLC was carried out using silica gel G (Merck) precoated readymade aluminium TLC plates. The extract to be analysed was diluted with the respective solvent and then spotted using a capillary tube. Various mobile phases were used for development and visualised under a UV lamp. Further, the developed plates were detected using various detecting agents like Dragendorff's, ninhydrin, Libermann-Burchard, conc. sulphuric acid and ferric chloride reagents[22].

High performance thin layer chromatography (HPTLC) profiling

HPTLC analysis was carried out with the best solvent system obtained from thin layer chromatography to detect the maximum number of components and peak abundance qualitatively and quantitatively. The extracts were dissolved in the respective solvents and 10 µL of the sample was applied on precoated Silica gel G (Merck) plates using Camag Linomat V applicator. The n-hexane and ethyl acetate extracts were eluted with Ethylacetate:n-hexane (6:4) while the ethanolic extract using methanol:ethyl acetate (1:1) and scanned under UV at 254nm/365nm. The data obtained were integrated through CAMAG software and the chromatographic fingerprints were developed for various extracts[22].

Selection of active extract

Radical scavenging activity by 1, 1,-Diphenyl-2-picrylhydrazyl assay (DPPH Assay)

The n-hexane, ethyl acetate and ethanolic extracts of *R. madagascariensis*, at different concentrations (50, 100, 150, 200, 250 µg/mL) were used for the study. Ascorbic acid (Sisco Research Laboratories Pvt Ltd) was used as the standard control. About 5 mL of crude extract or ascorbic acid was mixed with 3 mL of 0.004% DPPH solution and kept in dark for 30 minutes. Absorbance was measured against a blank at 517 nm using UV-Visible spectrophotometer (Shimadzu, Japan). Methanol with DPPH was used as a control. All the samples were tested in triplicate and the percentage radical scavenging activity was calculated using the formula,

$$\text{Percentage Radical scavenging Activity} = \frac{A-B}{A} \times 100$$

Where A = absorbance of the control (DPPH without the sample); B = absorbance of DPPH with the sample. The values were presented as Mean±SD of three replicates. Bar graphs were plotted with percentage scavenging activity against concentration and the IC_{50} values were calculated graphically using linear regression[23, 24].

Cell viability assay

Cytotoxicity screening on pancreatic cell lines was carried out using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide (MTT) assay. The basic principle involved is the ability of the metabolically active cells to convert yellow tetrazolium salt MTT to purple formazan crystals. Since the reduction occurs only in metabolically active cells, the extent of reduction is a measure of

cell viability. Cells (1×10^5/100 µL) were seeded in 96-well flat bottomed plates and incubated with various concentration of successive ethanolic leaf extract of *R. madagascariensis* for 48 hours. After the exposure period, the sample was removed and washed with phosphate-buffered saline (pH 7.4). Then 100 µL/well (5 mg/mL) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT), was added to each well and incubated for 4 hours at 37 °C. The formazan crystals were then solubilised by adding 100 µL of DMSO. The absorbance at 570 nm was measured with UV-Spectrophotometer using DMSO as a control blank. Percentage cell viability was calculated using the formula,

$$\text{Percentage cell viability} = \frac{\text{Absorbance of treated cells at 570nm}}{\text{Absorbance of control cells at 570nm}} \times 100$$

Bar graphs were plotted using the percentage cell viability at Y-axis and concentration of the sample in X-axis and the concentration required for a 50% inhibition (IC_{50}) was determined graphically[25-27].

Statistical analysis

All data were presented as the mean±standard deviations (SD). The experiment was repeated at least in triplicates. Student's *t-test* was included to compare the difference between groups using Microsoft Excel software. Any value of $p < 0.05$ was considered as statistically significant.

Results

The percentage yield after successive soxhlet extraction of the powdered leaves of *R. madagascariensis*, with n-hexane, ethyl acetate and ethanol was found to be 2.05, 3.55 and 9.79% w/w, respectively. Qualitative preliminary phytochemical analysis was performed initially with respective chemical reagents to detect the presence of phytoconstituents in the powder as well as various extracts. The n-hexane extract showed the presence of terpenoids, lipids and steroids whereas ethyl acetate extract showed positive results indicating the presence of alkaloids, phenols and steroids. Ethanol extract showed the presence of carbohydrates, flavonoids, saponins, alkaloids, tannins and glycosides. (Table 1).

TLC Profiling

Qualitative chromatographic analysis using TLC was performed to separate and identify the single or mixture of constituents in each extract. The development was carried out with various solvent systems at different ratios by trial and error method and the R_f values were tabulated in Table 2. Three spots were found to be active in n-hexane extract (0.62, 0.66, 0.86) whereas ethyl acetate exhibited four spots of R_f values 0.67, 0.73, 0.77, 0.79 in the solvent system chloroform:methanol (9:1). The ethanol extract showed two spots with R_f value 0.56 and 0.92 in the mobile phase system n-butanol:acetic acid:water (4:1:1).

HPTLC Profiling

HPTLC analysis was performed to detect the number of components and the peak abundance qualitatively and quantitatively at a higher resolution. (Fig. 1, 2) Ethyl acetate extract showed the maximum of 7 components in the solvent system Ethyl acetate:n-hexane (6:4) and the R_f values at 0.05, 0.09, 0.26, 0.36, 0.53, 0.60, 0.72. The n-hexane extract showed 4 components in the same solvent system with R_f values 0.05, 0.39, 0.51, 0.58 and percentage abundance peak area of 26.04, 19.12, 23.07 and 31.77. The ethanol extract in the mobile phase,

Table 1 — The percentage yield and phytoconstituents of successive extracts of leaves of *Ravenala madagascariensis* Sonn.,

Solvent used	Physical nature	Colour	Yield (%w/w)	Phytoconstituents
n- Hexane	Sticky	Light green	2.05% w/w	Terpenoids, lipids, steroids
Ethyl acetate	Semi-solid	Greenish black	3.55% w/w	Flavanoid, alkaloids, phenols, tannins, proteins, steroids
Ethanol	Semi-solid	Reddish brown	9.79%w/w	Flavanoids, glycosides, alkaloids, phenols, tannins, proteins, saponins

Table 2 — TLC profile of successive extracts of leaves of *Ravenala madagascariensis* Sonn.

Extract	Solvent system	No. of spots	UV detection & R_f values	
			Near UV 254nm	Far UV 365nm
n-Hexane extract	Chloroform:methanol (9:1)	3	No UV active compounds	0.62, 0.66, 0.86
Ethyl acetate extract	Chloroform:methanol (9:1)	4	No UV active compounds	0.67, 0.73, 0.77, 0.79
Ethanol extract	n- butanol:acetic acid:water (4:1:1)	2	No UV active compounds	0.56, 0.92

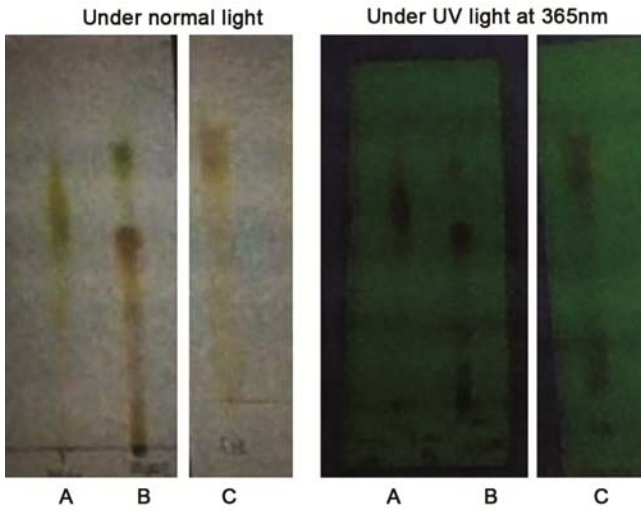

Fig. 1 — High Performance Thin Layer chromatography of successive extracts of leaves of *Ravenala madagascariensis* Sonn. under normal and UV (365nm) lamp (A- n-hexane extract, B-Ethylacetate extract, C-Ethanol extract)

methanol and ethyl acetate (1:1) showed 3 components with highest percentage abundance peak area of 64.83% at R_f value 0.69.

Radical scavenging activity by DPPH assay

The radical scavenging activity of successive extracts of *R. madagascariensis* at different concentration is shown in Fig. 3. All three extracts displayed dose-dependent scavenging of radicals. The scavenging effect of these extracts with IC_{50} values were in the order – ethanolic extract (52.21 µg/mL) > ethyl acetate extract (146.70 µg/mL) > n-hexane extract (254.83 µg/mL). The highest scavenging potential was exhibited by ethanolic extract compared to the standard Ascorbic acid which exhibited the highest antioxidant potential with the lowest IC_{50} value of 21.73 µg/mL and thereby was selected for the further cytotoxic studies.

Cell viability assay

The cell viability of PANC1 and SW1990 cell lines were evaluated by treating increasing concentrations of *R. madagascariensis*, extract at doses ranging from 0 to 250 µg/mL using MTT assay. The data on the percentage cell viability obtained after the exposure of cells to various concentrations of the ethanolic extract is given in Fig. 4 and 5. A dose-dependent decline in cell viability was observed in both the cell lines. The IC_{50} values were found to be 12.58 and 18.9 µg/mL in PANC1 AND SW1990 cells, respectively showing a higher cytotoxic effect in PANC1 adenocarcinoma cell lines compared to SW1990.

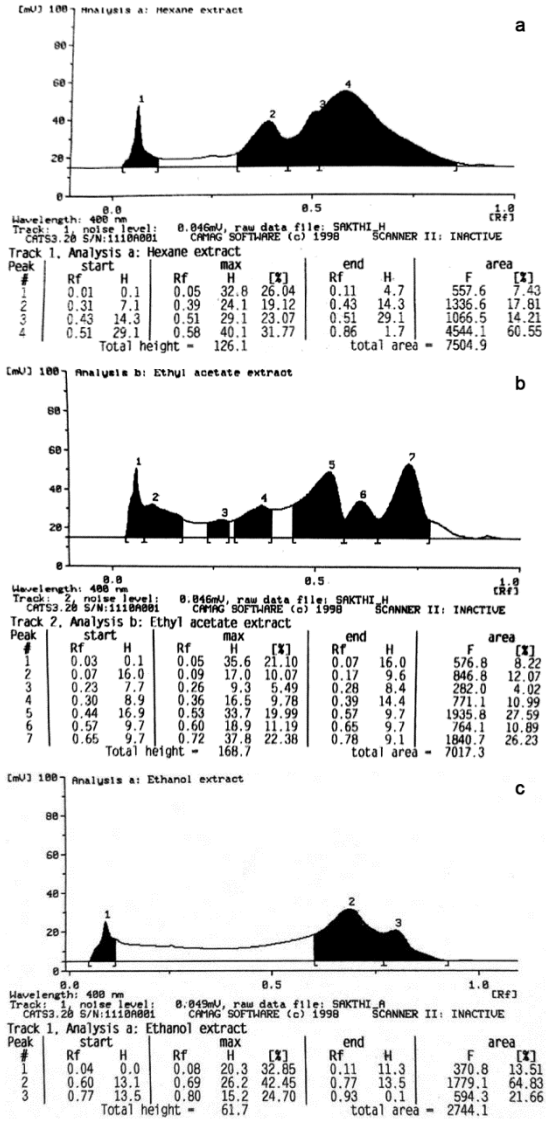

Fig. 2 — High Performance Thin Layer chromatography of successive extracts of leaves of *Ravenala madagascariensis* Sonn. (A-n-hexane extract, B-Ethyl acetate extract, C-Ethanol extract)

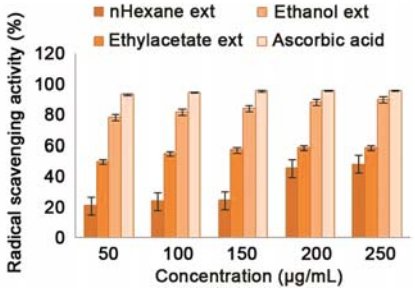

Fig. 3 — Radical scavenging activity of successive extracts of leaves of *Ravenala madagascariensis* Sonn., by DPPH assay. Plot of percentage radical scavenging activity against various concentrations of successive extracts. Data are expressed as mean±SD of triplicates. The IC_{50} value was found to be 254.83 µg/mL, 146.70 µg/mL, 52.20 µg/mL and 21.73 µg/mL for n-Hexane extract, Ethyl acetate extract, ethanol extract and Ascorbic acid repectively.

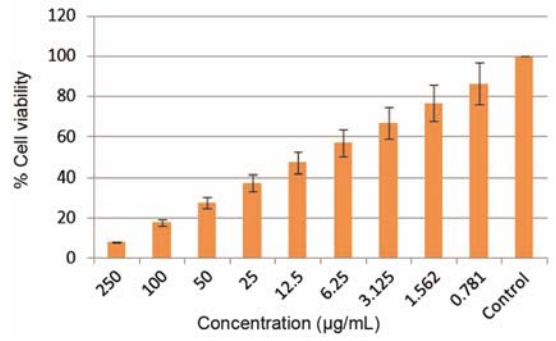

Fig. 4 — Effect of leaves of *Ravenala madagascariensis* Sonn., on PANC1 cell line using cell viability assay. Plot of percentage cell viability against various concentrations of successive ethanolic leaf extract for 48 h in PANC1 cells. 0.5% DMSO was used as control. Data are expressed as mean±SD of triplicates. The IC_{50} value was found to be 12.58 µg/mL.

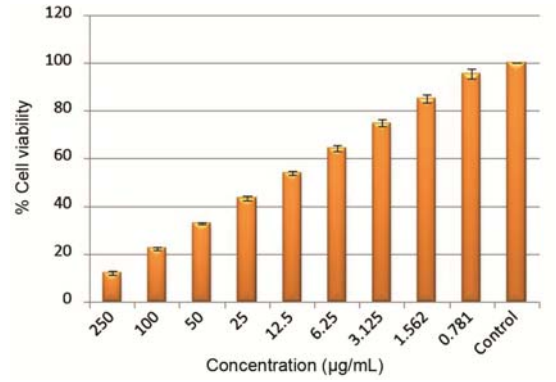

Fig. 5 — Effect of leaves of *Ravenala madagascariensis* Sonn., on SW1990 cell line using cell viability assay. Plot of percentage cell viability against various concentrations of successive ethanolic leaf extract for 48 h on SW1990 cells. 0.5% DMSO was used as control. Data are expressed as mean±SD of triplicates. The IC_{50} value was found to be 18.9 µg/mL.

Discussion

Allopathic medicine targets specific malignant molecular mechanisms whereas alternative medicine follows a holistic approach in treating the entire human body[28]. Insufficient preclinical and clinical data regarding the safety and efficacy of plant extracts remain a major drawback which in turn further demands serious investigations in this area of research. Several studies suggest that Type II diabetes occurs as a disease manifestation and a major prognostic factor for pancreatic cancer[13]. Molecular and genetic studies comparing diabetic patients at different stages exhibit molecular overlaps suggesting the relationship between pancreatic cancer and diabetes[29].

R. madagascariensis has been reported to be effective against diabetes and its associated disorders[18-20]. Its promising antioxidant potential and the presence of flavonoids led to further research on its anticancer activity[21]. In the present study, various extracts of leaves of *R. madagascariensis*, were prepared by successive soxhlet extraction using n-hexane, ethyl acetate and ethanol to extract the phytoconstituents according to their increasing polarity. The ethanolic extract showed the highest percentage yield of 9.79% w/w followed by ethyl acetate (3.55% w/w) and n-hexane (2.05% w/w).

The TLC and HPTLC profiling of all the successive extracts (Table 2 and Fig. 1, 2) provide a promising note on the presence and number of phytoconstituents. The R_f value of each phytoconstituent differs with different solvent systems. The selection of the solvent system for a particular extract can be achieved only by analysing the R_f values of compounds in different solvent systems[30]. The chromatographic profile of successive extracts provides valuable information on the nature, polarity and separation of compounds from the plant extract.

Excessive production of free radicals emerging beyond the control of a natural antioxidant defense system of the body results in oxidative stress-related disorders including cancer, ageing, cardiovascular and neurodegenerative diseases. The DPPH radical scavenging assay is one of the widely used assays which involves DPPH, a stable free radical with the absorbance maximum at 517 nm. DPPH becomes stable upon acceptance of an electron or hydrogen atom. The extract with antioxidant activity should possess hydrogen donating capacity which can convert the DPPH free radical into nonradical diphenyl picryl hydrazine turning purple to yellow[24,31,32]. The various successive extracts the leaves of *R. madagascariensis*, were screened for radical scavenging potential by DPPH assay. Among the successive extracts, the ethanolic extract was found to display stronger scavenging efficacy than the ethyl acetate and n-hexane extract as indicated by their IC_{50} values. The lower the IC_{50} value, the stronger is the scavenging activity as exhibited by the standard natural antioxidant Ascorbic acid.

Based on the radical scavenging potential the ethanolic extract of leaves of *R. madagascariensis*, was selected for its antiproliferative activity against pancreatic cancer. For the *in-vitro* cytotoxic studies two pancreatic cell lines PANC-1 and SW1990 were used. PANC-1 human pancreatic adenocarcinoma cell

line was selected based on their expression of multidrug resistance protein and poor sensitivity to Gemcitabine, the widely prescribed drug against pancreatic cancer[33-36]. Park *et al*., has reported Gemcitabine with an IC_{50} value of 10 nM in PANC-1 cell lines[37]. This report was also compared by Pak *et al.,* which involved a combinational therapy of a herbal mixture extract (H3) exhibiting an IC_{50} value of 0.05 mg/mL along with Gemcitabine (3 nM) showing a better cytotoxic activity than H3 the extract alone (0.07 mg/mL)[38]. In another study carried out by Yue *et al*., Gemcitabine was reported with an IC50 value of 0.079 µg/mL in SW1990 cell line[39]. In the present investigation, it was found that the cytotoxic effect of ethanolic extract of *R. madagascariensis*, on PANC-1 and SW1990 was remarkable and with almost close IC_{50} values of 12.58 and 18.9 µg/mL. The study is in accordance with the American Cancer Institute which states a drug to be a promising candidate if the EC_{50} value of a crude extract is less than 30 µg/mL[40,41]. Thus the study validates the *in-vitro* antitumor activity of the successive ethanolic leaf extract against two human pancreatic cell lines PANC1 and SW1990 which can be attributed to the presence of flavonoid enriched phytoconstituents.

Conclusion

In the present work, the phytochemical, radical scavenging and cytotoxic activity of successive extracts of leaves of *R. madagascariensis* were screened. Preliminary phytochemical screening revealed the presence of various phytoconstituents including terpenoids, lipids, steroids, alkaloids, flavonoids, saponins, tannins and glycosides. TLC and HPTLC were carried out. All the three successive extracts, the n-hexane, the ethyl acetate and ethanolic leaf extracts were subjected to free radical scavenging activity. The ethanolic extract was found to possess the highest antioxidant activity with IC_{50} value of 52.21 µg/mL. Hence, the ethanolic extract was selected for the further *in-vitro* anti-cancer cell line studies. The ethanolic extract of leaves of *R. madagascariensis* showed promising cytotoxic activity in PANC-1 and SW1990 cell lines with an IC_{50} value of 12.58 and 18.9 µg/mL, respectively against pancreatic cancer. The study strongly suggests the ethanolic extract can turn out to be an effective flavonoid-rich extract against oxidative damage and pancreatic cancer. Thus, the current work provides valuable data for further studies that can be aimed at exploring the phytoconstituents responsible for the biological activity and the biochemical mechanisms involved in it.

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