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Passiflora Edulis Leaf Extracts Towards Drug Resistant Cancer Cell

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Abstract - **Passiflora edulis, commonly known as passion fruits is a vine species of passionflower. It is cultivated commercially in tropical & subtropical areas for its sweet, seedy fruit. Fruit is berry type, oval to round, at maturity it is yellow to dark purple with juicy interior filled with numerous seeds. Passiflora Edulis L., Passifloraceae, leaves were collected in the city of Rajahmundry Andhra Pradesh, India. The leaves were air-dried at 45- 50 °C for 72 hours to 80hours, powdered, and stored at room temperature. Cytotoxicity of the studied samples in this study, we first screened the cytotoxicity of crude extracts belonging to plassiflora edulis plant towards drug-resistant cancer cells. The results are shown in Table 1. Plassiora Edulis plant extracts with IC50 values below of 20 µg/mL following incubation between 48 and 72 h are recognized as potential cytotoxic substances. In the present study, multi-factorial drug-resistant cancer cell lines such as leukemia. In this study, Passiflora edulis had good cytotoxicity against CCRF–CEM leukemia cells and its resistant subline CEM/ADR5000 cells. Their selectivity to these two cells lines, indicates that they can be sources for the development of novel anticancer drugs to fight leukemia.**

Index Terms - **PEL (Passiflora edulis leaves), PEF (Passiflora edulis fruit), PEP (Passiflora edulis Pericarp).**

Abbrevations:
PEL: Passiflora edulis leaves
PEF: Passiflora edulis fruit
PEP: Passiflora edulis fruit pericarp
IC50: inhibitory concentration 50 %

INTRODUCTION

Passiflora edulis, commonly known as passion fruits is a vine species of passionflower. It is cultivated commercially in tropical & subtropical areas for its sweet, seedy fruit. Fruit is berry type, oval to round, at maturity it is yellow to dark purple with juicy interior filled with numerous seeds. The flavor of the juice is slightly acidic & musky. The passion fruit flavor can be compared to guava fruit.It belongs to Kingdom- Plantae, Class- Rosids, Order- Malphighiales, Family- Passifloraceae, Genus – passiflora , species - P.edulis. The plants belonging to this genus have been used in traditional medicine for a variety of conditions such as gastrointestinal conditions, neurological complications, cardiovascular conditions, inflammation & anxiety. These uses were attributed to presence of several active compounds such as phenolic compounds, alkalides, flavanoids & saponins. Passion fruit recived its name from Latin word “Passio” which means passion, or suffering. Around 1700 name was given by missionaries in Brazil as an educational aid while trying to convert the indigenous inhabitants to Christianity; its name was Flor das cinco chagas or flower of the five wounds to illustrate the crucifixon of Christ.

The leaves of P.edulis alcohol extract contain cycloartane triterpenoid saponins that include cyclopassifloside and & six other cycloartane triterpenoids. The extract also contains cyclopassifloside and which exhibited antidepressant like effect when administered orally in mice. the hydroethanolic extract showed the presence of flavonoids which exhibited antioxidant & wound healing activities. Different types of leaf extracts were obtained using hexane, water, ethyl acetate, methanol, methanol and these extracts showed antibacterial activity which was attributed to presence of compounds such as glycosides, flavonoids, alkaloids, phenols, resins and balsam. The phytochemicals such as saponnins, steroids, tannins, alkaloids, flavones obtained from different types of leaf extracts have antimicrobial activity against different bacteria. The ethanolic extracts were found to have maximum antimicrobial potential against E.Coli.it is also used to

treat epilepsy, ulcers & haemorrhoids, it also exhibit potential antioxidant activity.

Passiflora Edulis Leaves (PEL)

MATERIALS AND METHODS

PLANT MATERIAL

Passiflora Edulis L., Passifloraceae, leaves were collected in the city of Rajahmundry Andhra Pradesh, India. The leaves were air-dried at 45- 50 °C for 72 hours to 80hours, powdered, and stored at room temperature.

Chemicals

Ethanol, acetonitrile and sodium hydroxide were purchased. Methanol and hydrochloric acid were obtained. All solvents of analytical reagents are graded, which were of HPLC grade. Water was purified. The standards vitexin, isovitexin, orientin and isoorientin were obtained.

Experimental design for extraction process

Percolation was chosen as the extraction method of extraction process, and the particle size of the dried plant material used to make the extractions was moderately coarse (WHO, 2011). Three factors at three levels were evaluated, to work out their influence on the ultimate concentration of TF (drug: solvent ratio, at 1:10; 1:15 and 1:20, w/v; ethanol concentration, at 25, 50 and 75% and extraction times of 24, 48 and 72 h). The ethanol extracts was evaporated under reduced pressure at 40 °C. The remaining water was eliminated by freeze-drying and the dry extracts were stored at 4 °C until HPLC quantification. Aqueous extract from P. edulis leaves was obtained by infusion according to previous works (Costa et al., 2013). The aqueous extracts were filtered and freeze-dried.

Determination of the total flavonoids (TF) content

The total flavonoids of P. edulis and its degradation products were quantified by an HPLC- method adapted from previous investigations by our group (Costa et al., 2013). A Shimadzu Liquid Chromatography System equipped with a DGU-20 degasser, LC-6AD binary pumps, SPD M20-A DAD detector was used for these analyses. The data were processed using Labsolution software®. The experiments were administered on a reverse-phase Phenomenex Luna C18 column (250 mm × 4.6 mm i.d. 5 μm), maintained at 30 ± 1 °C. The mobile phase consisted of a liner gradient of phase A (water: acetonitrile: ethanoic acid , 90:10:1 v/v/v) and phase B (acetonitrile :water: ethanoic acid , 90:10:1 v/v/v) in two steps: 11% B (0–5 min), then 11–15% B (5–20 min). The flow rate was kept constant at 1 ml/min. The mobile phase was prepared and degassed by sonication daily before use. The chromatogram was monitored at 340 nm, and UV spectra of individual peaks were recorded within the range of 200–450 nm. The samples were prepared by dissolving the lyophilized crude extracts in methanol: water (1:1, v/v) and filtering through a 0.45 μm PVDA membrane before injection. The concentration of the sample extracts was 1 gm/ml. Vitexin was employed as standard and Total Flavonoids were quantified by the sum of all the chromatographic signals identified as flavonoids by their UV-DAD spectra, being expressed as mg-eq vitexin/g dry extract.

Stability study under stress conditions

Stability studies under neutral, acid and alkali hydrolysis, oxidative and photolytic conditions performed by adaptation to the methodology reported by Singh and Bakshi (2000). Briefly, 10 mg of the extract was subject to different hydrolytic, oxidative and photolytic conditions. The condition at which the TF content decreases by between 20% and 80% was used to classify the extract as extremely labile, very labile, labile, stable, very stable or practically stable. In order to raised understand the extract stability, besides TF quantification, the variation on each C-glycosyl flavonoid and degradation product decided. The content of individual compounds was expressed as relative amount (%), according to Equation 1. Relative amount (%) =Area of the peak of the individual flavonoid All flavonoid peaks total area × 100.

Evaluation of sedative activity

The sedative activity of the extract was evaluated within the ethyl ether-induced hypnosis test following the methodology reported by Gazola and associates (2018). Male adult Swiss ICR mice (age: 10–12 weeks and 30 g weight approximately) were used. The animals were supplied by the animal house of the Department of Pharmacy of the Vikas Institute of Pharmaceutical Sciences and were kept under constant temperature conditions (22 °C ± 1), 12 h light/dark cycles, with food and water ad lib. The assays were administered in accordance with the international and native ethical guidelines on the utilization and care of laboratory animals, and approvingly of the local Research Ethics Committee (Act 02/2016 Faculty of Science).

The test was carried out with four groups of animals (n = 10 animals per group). Group I: vehicle (distilled water). Group II: Diazepam as positive control (1 mg/kg dissolved in distilled water). Group III: aqueous extract (60 mg/kg suspended in distilled water) and Group IV: the optimized hydroalcoholic extract (60 mg/kg suspended in distilled water).

After 12 h fasting, all treatments got orally (0.1 ml/mg) 1 h before the beginning of the assay, apart from the positive control, which was given 30 min earlier. After this point, each animal was placed during a glass chamber (previously saturated with ether for five min). Once the animal lost postural reflex, it had been far away from the chamber and placed within the supine position. The time until the animal resumed the ventral position was recorded. For analysis of the info on pharmacological sedative activity, the software GraphPad Prism Version 7.0 was used, and one-way ANOVA was applied, followed by the Dunnett's test (95%).

RESULT

Cytotoxicity of the studied samples in this study, we first screened the cytotoxicity of crude extracts belonging to plassiflora edulis plant towards drug-resistant cancer cells. The results are shown in Table 1 . All tested extracts had IC50 values below 80 µg/ mL. Ten extracts and Passiflora edulis leaves (PEP) and fruit (PEF) displayed IC50 values below 20 µg/mL in CCRF–CEM cells (Table 2). These extracts were further selected for IC50 determination towards a panel of sensitive and MDR cell lines. The results summarized in Table 3 indicate that all selected extracts were also active against P-glycoprotein-overexpressing CEM/ADR5000 leukemia cells with IC50 values below 40 µg/mL. IC50 values ranged from 10.13 µg/ mL (towards CEM/ADR5000 cells) to 72.01 µg/mL (on resistant colon carcinoma HCT116 (p53−/−) cells) for PSR, from 14.97 µg/mL (on CEM/ADR5000 cells) to 65.68 µg/mL (against HCT116 (p53−/−) cells) for PSB, from 18.21 µg/mL (against CEM/ADR5000 cells) to 65.21 µg/mL (on HCT116 (p5+/+) cells) for PSL and from 0.11 µg/mL (towards CCRF–CEM cells) to 108 µg/mL (against CEM/ADR5000 cells) for doxorubicin in the 8 other cancer cell lines studied. Apart from extract from P. staudtii, other extracts were less active on carcinoma cells including normal AML12 hepatocytes, with IC50 values above 80 µg/mL. Collateral sensitivity (or hypersensitivity: higher toxicity to resistant than to sensitive cells with a degree of resistance below 1) (Kuete et al. 2013a) was observed in CEM/ADR5000 cells to PSB (degree of resistance of 0.87-fold) and PSR (0.59-fold) (Table 3). Hypersensitivity of resistant carcinoma cells was also recorded in many cases to PSL, PSB or PSR even though they were moderately active. However, if crossresistance of CEM/ADR5000 cells to the tested extracts were observed, the degrees of resistance were in all cases lower than that of doxorubicin (Table 3). PEL, PEP and PEF had IC50 values below 1 and 10 µg/mL in sensitive CCRF/CEM cells and it resistant subline CEM/ADR5000 cells respectively; they were subsequently selected for mechanistic studies.

Cell cycle distribution and apoptosis the best extracts (PEL, PEP and PEF) as well as doxorubicin were used to treat CCRF–CEM cells at their IC50 values, and the cycle distribution was analyzed.

Results depicted in Fig. 1 show dose-dependent and significant modifications of the cell cycle phases after treatment of cells with all samples. Both PEF and PEL induced cell cycle arrest in G0/G1 phase while PEP induced cell cycle arrest in S-phase. After treatment with these three extracts, CCRF–CEM cells underwent apoptosis with dose-dependent increases in sub-G0/G1 phase. The percentages of cells in sub-G0/G1 phase varied from 9.31 % (in 24 h) to 48.69 % (72 h), from 8.87 % (in 24 h) to 33.98 % (72 h) and from 11.03 % (24 h) to 21.63 % (72 h) after PEP, PEL and PEF treatments respectively, while doxorubicin increased apoptosis in a range of 6.02 % (24 h) to

51.87 % (72 h). The highest percentage of subG0/G1 phase in non-treated cells was only 6.42 % after 72 h.

Effects on the activity of caspases, MMP and ROS

After treating CCRF–CEM cells for 6 h at different concentrations of PEF, PEL and PEP, no changes of caspase 3/7, caspase 8 and caspase 9 activities were observed. No increase in ROS production was also not found in CCRF–CEM cells treated with the three extracts (data not shown). PEF, PEL and PEP induced significant MMP loss in the respective ranges of 35.3 % (1/2-fold IC50 treatment) to 46.7 % (2-fold IC50), 28.2 % (1/2-fold IC50) to 53.8 % (2-fold IC50) and 36.6 % (1/2-fold IC50) to 51.0 % (2-fold IC50) (Fig. 2). A 48.6 % loss of MMP at 2-fold IC50 of vinblastine was previously reported under similar experimental conditions in CCRF–CEM cells (Kuete et al. 2013a).

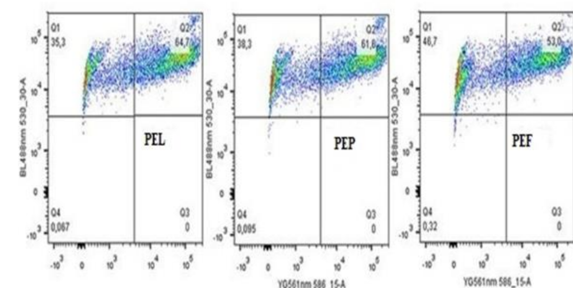
Species (family)	Traditional uses	Parts used (%yield)	Bioactive or potentially bioactive components	Bioactivity of crude extract
Passiflora edulis Sims (Passifloraceae);	Treatment of cancer, fungal infections, inflammation, insomnia and anxiety, antihypertensive (Ichimura et al. 2006), gastric trouble (Silva et al. 2006), antioxidant (Kannan et al. 2011)	Fruit (3.92 %); fruit pericarp (2.73 %), leaves	vitexin, isovitexin, orientin and isoorientin	Antimicrobial activities of methanol extract against

Cytotoxicity of methanol extracts of, Passiflora edulis of drug-resistant cancer cell lines

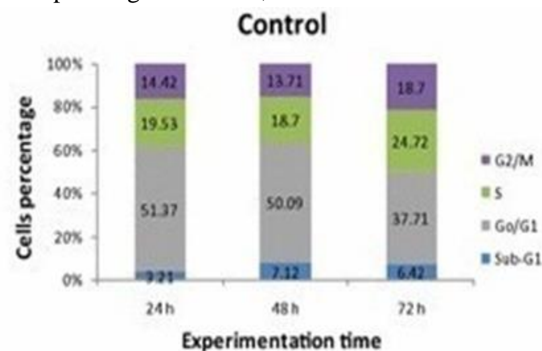
Plant	Parts	IC_{50} values (µg/mL)
Passiflora edulis	Fruit pericarp (PEP) Fruit (PEF) Leaves (PEL)	*3.41 ± 0.55* *0.69 ± 0.13* 36.28 ± 2.84

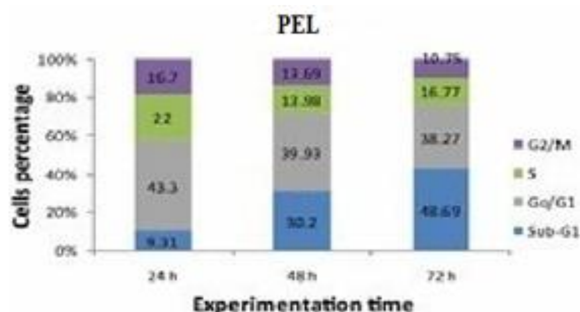
Cell Lines	PEF	PEP	PEL
CEM/ADR5000	*8.20 ± 1.02* (11.88)	*18.40 ± 1.42* (5.40)	*18.21 ± 1.45* (1.34)
MDA-MB-231-*pcDNA*	>80	>80	52.08 ± 4.98
MDA-MB-231-*BCRP* Degree of resistance	>80	>80	61.98 ± 4.31 (1.19)
HCT116 ($p53^{+/+}$)	>80	>80	65.21 ± 7.15
HCT116 ($p53^{-/-}$) Degree of resistance	>80	>80	56.97 ± 4.09 (0.87)
U87MG	>80	>80	65.21 ± 5.79
U87MG.Δ*EGFR* Degree of resistance	>80	>80	68.65 ± 3.48 (1.05)
HepG2	>80	>80	46.98 ± 3.17 (>1.70
AML12 Degree of resistance	>80	>80	>80

The degree of resistance was determined as the ratio of IC_{50} value in the resistant divided by the IC_{50} in the sensitive cell line; CEM/ADR5000, MDA-MB-231-*BCRP*, HCT116 ($p53^{-/-}$), U87MG.Δ*EGFR* and AML12 were used as the corresponding resistant counterpart for CCRF–CEM (Table 1), MDA-MB-231-*pcDNA*, HCT116 ($p53^{+/+}$), U87MG and HepG2 respectively; the tested methanol extracts of PEF: *Passiflora edulis* fruit; PEP: *Passiflora edulis* fruit pericarp; and PEL: *Passiflora edulis leaves.*



Effect of PEF, PEL and PEP on the mitochondrial membrane potential in CCRF–CEM cells. *C* control; PEL was tested at 24 h at 0.35 µg/mL (PEL1), 0.69 µg/mL (PEL2), and 1.38 µg/L (PEL3) while was tested at 0.29 µg/mL (PEF1), 0.57 µg/mL (PEF2), and 1.14 µg/mL (PEF3) and was tested at 0.18 µg/mL (PEP1), 0.36 µg/mL (PEP2), and 0.72 µg/mL (PEP3) corresponding to 1/2-fold, IC_{50} and 2-fold IC_{50}. Data.





Cell cycle distribution of CCRF–CEM leukemia cells treated with extracts from PEF, PEL and PEP. PEF, PEL and PEP were tested at 0.69, 0.57 and 0.36 and 8.02 µg/mL respectively while corresponding to their IC_{50}

DISCUSSION

Plassiora Edulis plant extracts with IC_{50} values below of 20 µg/mL following incubation between 48 and 72 h are recognized as potential cytotoxic substances. In the present study, multi-factorial drug-resistant cancer cell lines such as leukemia CEM/ADR5000 cells over-expressing P-gp, breast adenocarcinoma MDA-MB-231-*BCRP* clone 23 expressing BCRP, EGFR-transfected U87MG.Δ*EGFR* glioblastoma cells and p53 knockout HCT116 (*p53* $^{-/-}$) colon cancer cells were used to determine the cytotoxicity the selected plant extracts. In the first step of the investigations, we carried out preliminary assays with the sensitive leukemia CCRF–CEM cells. In regard to the NCI threshold of PEL, PEP and PEF (Table 2) displaying IC_{50} values below 20 µg/mL were selected and further tested on a panel of 8 other cell lines. Interestingly, the P-gp over-expressing leukemia CEM/ADR5000 was also sensitive to most of the extracts with IC_{50} value below 20 µg/mL obtained with PEL, PEF and PEP. This suggests that these extracts can be used to manage hematological cancers including resistant phenotypes. Data obtained with PEL, PEF and PEP are very interesting as they displayed IC_{50} values below 10 µg/mL in the resistant CEM/ADR5000 cells and even below 1 µg/mL in its sensitive counterpart CCRF–CEM cells. Nonetheless, they were not active in carcinoma cells, clearly indicating their selectivity to leukemia cells. Alteration of MMP has been reported as a mode of apoptosis induction of plant extracts (Kuete and Efferth 2015). PEL, PEF and PEP induced MMP loss but no caspase activation nor increase ROS production. Hence, MMP is the main mode of induction of apoptosis of PEL, PEF and PEP in CCRF–CEM cells as observed in this study. *Passiflora edulis* towards the cell line panel tested in this study is being reported for the first time. Also, the methanol extracts of the leaves and fruits of *Passiflora edulis* harvested in India were screened at 100 µg/mL against HCT-116 cells, HepG2 cells as well as against the breast carcinoma MCF-7 cells and lung carcinoma A-549 cells; As results, less than 50 % growth inhibition was recorded.

CONCLUSION

In this study, *Passiflora edulis* had good cytotoxicity against CCRF–CEM leukemia cells and its resistant subline CEM/ADR5000 cells. Their selectivity to these two cell lines, indicates that they can be sources for the development of novel anticancer drugs to fight leukemia. PEF, PEL & PEP were the most cytotoxic extracts and induced apoptosis in CCRF–CEM cells mediated by loss of MMP. Further phytochemical investigations of these extracts will be done to isolate their active constituents.

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