IN VIVO ANTITUMOR, ANTIOXIDANT ACTIVITIES AND TOXICITY PROFILE OF ETHYL ACETATE CRUDE LEAF EXTRACT OF *PARKINSONIA ACULEATA* L. (FABACEAE) ON B16F10 MELANOMA

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ABSTRACT

The present study evaluates the effect of ethyl acetate crude leaf extract of *Parkinsonia aculeata* L. (Fabaceae) on B16F10 mice melanoma. Crude extract was tested for Acute Oral Toxicity and Sub acute toxicity studies. The crude extract was then taken for *in vivo* antitumor study on B16F10 melanoma induced BALB/c mice. Melanoma was induced s.c. using B16F10 cell suspension of 5×10^5 cells/animal in 18 animals randomized into Group 1 as positive control (Vehicle), Group 2 treated with 100 mg/ kg bwt. and Group 3 treated with 300 mg/ kg bwt. extract. During the treatment the size of implanted tumors were measured and tumor volume calculated. Sub acute toxicity studies showed that the extract does not possess any cytotoxicity and treatment with crude extract reduced the tumor growth in a dose and time dependent manner. P values of < 0.05 were considered significant for $N = 6$. The ethyl acetate crude leaf extract contain the active principles that mediate the anticancer property. The active components that mediate the biological property may be majorly flavonoids viz. apigenin, vitexin, iso-vitexin, orientin, iso-orientin and chrysoeriol. Further work is needed to ascertain the mechanism of actions of these flavonoids. The study also provides the safety profile and dosage of the crude leaf extract. Thus the plant's leaves could be good source of natural and potent chemotherapeutic.

Keywords: melanoma, cytotoxicity, *Parkinsonia aculeata* L., flavonoids, antitumor

INTRODUCTION

Cancer is the second leading cause of death. Based on the years lost to cancer, melanoma would merit a higher ranking because relatively young people are affected by this malignancy¹⁻³. The WHO has estimated that approximately 80 % of the world's population depends on traditional medicines for meeting their primary health care needs⁴. In spite of advances in medicine, still there is no reliable cure for melanoma. So this deadly disease demands attention from investigators worldwide. Increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extraction and development of several drugs and chemotherapeutics from these plants as well as from traditionally used rural herbal remedies⁵. Medicinal plant drugs can be placed into two broad categories. Firstly, they are included in complex mixtures containing a wide variety of compounds and secondly they are used as pure, chemically defined active principles⁶. Chemotherapy, being a major treatment modality used for the control of advanced stages of malignancies and as a prophylactic against metastasis, exhibits severe toxicity on normal tissues⁷. The ethnopharmacological approach for the search of new anticancer agents from the plant sources has proved to be more predictive. There are many natural substances which exhibits antitumor activities. *Parkinsonia aculeata* (Fabaceae) is commonly known as “Vilayti Kikar, Jerusalem Thorn” and widely cultivated throughout world. Very previous reporting indicated the presence of various flavonoids in leaves but since then there has been no such work reported on the exploration of its medicinal importance⁸. Flavonoids are usually present in dietary intakes and also reported to possess anticancer potential thus the plant's leaves were taken for study.

MATERIAL AND METHODS

Collection of plant material

The leaves of *Parkinsonia aculeata* L. were collected from areas around Hoshangabad and Bhadbhada in Bhopal, (M.P.), India. The verification and identification of the herbal species from Bhopal region was done by the Botanist Dr. Jagriti Tripathi, Unique College, Barkatullah University, Bhopal, India. The leaves of the authenticated herbal plants were then collected, washed in running tap water and dried for 20 days to obtain constant weight. The dried sample was ground into fine particles with a grinder. The powdered sample was kept in airtight plastic containers for further work.

Crude Extraction

Crude extraction was carried according to Harborne⁹. Powdered leaves weighing 500 g were extracted in Soxhlet apparatus with successive extraction in 500 ml of Petroleum Ether and Ethyl Acetate (Et O Ac). The extraction was done for 48 h in each solvent and then filtered through Whatmann filter paper No. 1. The extracts were concentrated to about 1/6th of the original volume at 60°C under reduced pressure rotary vacuum evaporator. The extracts were then air dried for 3 weeks to a constant weight and kept in airtight vials (Borosil) for further work.

Phytochemical analysis of crude extracts

The phytochemical analysis was carried out using procedures of Sofowora¹⁰; Trease and Evans¹¹.

Thin layer chromatography

The crude extract was further assessed on TLC (E Merk F₂₅₄) plates for initial identifications of flavonoids according to Mabry¹²; Harborne⁹.

In-vivo Antitumor study against B16F10 mice melanoma cell lines

The *in vivo* study was performed according to Bernward *et al*¹³. Before going on to antitumor study acute oral toxicity was tested for the ethyl acetate crude extract of *Parkinsonia aculeata* L. leaves.

Acute Oral Toxicity

Acute oral toxicity study was performed out according to OECD (Organization for Economic Co-operation and Development) 423, guideline for testing of chemicals (OECD, 2001). Animals were fasted prior to dosing and water was withheld for 3-4 hours only. Following the period of fasting the animals were weighed and the crude extract administered. Three animals were used at each step. The dose level used was selected at 300 mg/kg bwt. (Annexure 2c) followed by 2000 mg/kg bw (Annexure 2d). Distilled water was given to other group as a control. Animals were observed individually after dosing once during the first 30 minutes, periodically during the first 24 h with special attention given during the first four hours and daily thereafter for 14 days for clinical signs like behavioral profile.

Sub-acute toxicity study

The study was carried out as per OECD guideline for testing for chemicals. IAEC number for the present study was [PBRI/11/IAEC/PN-182/A]. Mice were randomly divided into three groups of 6. The extract was prepared at concentrations of 100 and 300 mg/kg bodyweight while PEG was given to control group. Repeated dosing was continued till 28 days. At the end of experiment blood was collected from ocular orbital puncture for biochemical estimations to study blood, glucose profile, liver function test, lipid profile and kidney function test. The serum was separated by centrifugation at 15000 rpm on 4°C. Biochemical parameters were examined using Autoanalyzer (Star 21 Span Diagnostic). After blood collection internal organs liver and kidney were examined for histological studies.

Induction of tumors in mice

Cell suspension of 5×10^5 cells/animal was implanted s. c. at the shaved part. The mice bearing the tumors were randomly divided into 3 groups with 6 mice in each group. The dosing was started when tumors had reached a mean diameter of 6 mm and this day was designated as Day 0.

Experimental design

Mice (n = 18) were randomized into following three groups: Group 1- Kept as Positive Control (Vehicle) treated with (0.2 ml PEG). Group 2- Treated with 100mg/kg bwt. ethyl acetate extract of *Parkinsonia aculeata* L. leaves (p.o.) [D₁]. Group 3- Treated with 300 mg/kg bwt. ethyl acetate extract of *Parkinsonia aculeata* L. leaves (p.o.) [D₂].

Evaluation of tumor growth

During the treatment the size of implanted tumors was measured by Vernier Calliper to construct the tumor growth curve. The vehicle was kept on normal diet with PEG only. The test group D1 and D2 was given doses throughout the experiment daily orally. Tumor volume was calculated by the formula:

$$\text{Tumor Volume} = \text{Length} \times \text{Width}^2 \times 0.5$$

In vivo screening of antioxidant profile**Lipid Peroxidation (LPO)**

It is an autocatalytic process which is a common consequence of cell death. This process may cause peroxidative tissue damage in inflammation and cancer. The concentration of TBARS was measured spectrophotometrically using TBA reagent according to procedure¹⁴. Briefly, one volume of homogenate was mixed with 0.5 volume of trichloroacetic acid (15 %w/v) and centrifuged at 200 X g for 10 minutes. One ml of the supernatant was mixed with 0.5 ml TBA (0.7 %w/v) and boiled for 10 minutes. After cooling the absorbance was recorded at 535 by spectrophotometer (Sistronix 2202). MDA concentration was calculated using extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

Reduced Glutathione (GSH)

GSH is naturally occurring substance that is abundant in many living creatures. Its deficiency can lead to tissue disorder and injury. The procedure was followed according to Ellman *et al*¹⁵. To measure the GSH level the tissue homogenate (in 0.1 M phosphate buffer pH 7.4) was taken. The homogenate was added with equal volume of 20 % trichloroacetic acid (TCA) containing 1 mM EDTA to precipitate the tissue proteins. The mixture was allowed to stand for 5 minutes at 2000 rpm. The supernatant (200 µl) was then transferred to a new set of test tubes and added 1.8 ml of Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid) (0.1 mM was prepared in 0.3 M phosphate buffer with 1 % of sodium citrate solution). Then all the test tubes make up to the volume of 2 ml. After completion of the total reaction solutions were measured at 412 nm against blank. Absorbance values were compared with standard curve generated from known GSH.

Superoxide Dismutase (SOD)

SOD is metalloprotein and is the first enzyme involved in the antioxidant defense by lowering the steady-state level of O₂. It was performed according to the method of Marklund¹⁶. Assay mixture contained 0.1 ml of sample 1.2 ml of sodium pyrophosphate buffer (pH 8.3, 0.052 M), 0.1 ml phenazine methosulphate (186 µM), 0.3 ml of 300 µM nitrobluetetrazolium, 0.2 ml NADH (750 µM). Reaction was started by addition of NADH. After incubation at 30°C for 90 s, the reaction was stopped by the addition of 0.1 ml of glacial acetic acid. Reaction mixture was stirred vigorously with 4.0 ml of n-Butanol. Mixture was allowed to stand for 10 minutes, centrifuged and butanol layer was separated. Color intensity of chromogen in the butanol layer was measured at 560 nm spectrophotometrically and concentration of SOD was expressed as units/mg/protein.

Catalase (CAT)

CAT is a hemeprotein, localized in the peroxisomes. This enzyme catalyzes the decomposition of H₂O₂ to water and oxygen and thus protecting the cell from oxidative damage. It is a key component of antioxidant defence system. The assay was performed according to Maehly¹⁷. Catalase activity was measured by the method of Aebi¹⁸. 0.1 ml of supernatant was added to cuvette containing 1.9 ml of 50 mM phosphate buffer (pH 7.0). Reaction was started by the addition of 0.1 ml of freshly prepared 30 mM H₂O₂. The rate of decomposition of H₂O₂ was measured spectrophotometrically from changes in absorbance at 240 nm. Activity of catalase was expressed as units/mg protein.

Table 1: Phytochemistry of different crude extracts of *Parkinsonia aculeata* L. leaves

S. No.	Compound	Petroleum ether extract	Ethyl acetate extract
1.	C-Glycosides	-	+++
2.	Terpenes	++	+
3.	Flavonoids	-	+++
4.	Alkaloids	-	-
5.	Saponin	-	-
6.	Phenolic Compounds	-	+
7.	Gums and Mucilages	-	-
8.	Reducing Sugars	-	+

(-) absent; (+) present; (++) strongly present; (+++) very strongly present

Table 2: Sub acute toxicity of ethyl acetate crude extract of *Parkinsonia aculeata* L. leaves on BALB/c mice

Treatment Group	GLU mg/dL	BUN mg/dL	CR mg/dL	TG mg/dL	SGOT U/L	SGPT U/L	ALP U/L	BIL mg/dL	Chol mg/dL
Vehicle	112.00 ± 11.90	32.17 ± 5.23	0.99 ± 0.08	192.67 ± 9.42	40.83 ± 6.46	38.67 ± 5.50	119.33 ± 11.33	0.50 ± 0.10	199.50 ± 11.48
Extract	104.00 ± 9.96	31.17 ± 5.15	0.80 ± 0.05	188.00 ± 7.46	38.17 ± 6.18	40.17 ± 4.22	118.67 ± 6.74	0.82 ± 0.04	189.33 ± 12.04

GLU-Glucose; BUN-Blood Urea Nitrogen; CR-Creatinine; TG-Triglycerides; SGOT- Serum glutamic oxaloacetic transaminase ; SGPT- Serum glutamic pyruvic transaminase; ALP-Alkalinephosphatase; BIL-Bilirubin; Chol-Cholesterol

Table 3: Showing statistical analysis of *in vivo* antitumor activity of ethyl acetate crude leaf extract of *Parkinsonia aculeata* L.

Group	Day 2 (mm ³)	Day 4 (mm ³)	Day 6 (mm ³)	Day 8 (mm ³)	Day 10 (mm ³)	Day 12 (mm ³)	Day 14 (mm ³)
Positive Control	297 ± 30.38	560.7 ± 20.1	904.5 ± 34.96	1325.7 ± 39.87	307.2 ± 192.2	4458.5 ± 107.1	5173.2 ± 88.25
D1 (100mg/ kg bwt..)	317.2 ± 20.87	488 ± 21.7	656.2* ± 20.99	1130.7 ± 27.95	2278.8 ± 90.8	3068* ± 110.78	3057.2 ± 429.9
D2 (300mg/ kg bwt..)	326.3 ± 5.85	483.7 ± 15.93	579.2** ± 14.39	943.17*** ± 32.97	2046.3** ± 189.98	3003.5* ± 213.98	2840** ± 156.3

Data are expressed as Mean ± SEM. Statistical analysis was done by One Way ANOVA followed by Dunnett's Multiple Comparison Test, *P < 0.05, **P < 0.01, ***P < 0.0001 as compared to control (n = 6)

Figure 1: TLC of ethyl acetate crude leaf extract of *P. aculeata* L. in Et O Ac: MeOH: H₂O (a) (100:16.5:13.5) (b) (70:15:5) **Figure 2: Histopathological examinations of Kidney and Liver while sub acute toxicity study**

Statistical analysis

The results are presented as means $\pm$ S.D. Significant differences between control and treatment groups were calculated using One way ANOVA followed by Dunnett's multiple comparison test. Number of replicates (N) = 6. P values of < 0.05 were considered significant (Table 2). The analysis was performed using Graphpad Prism 6 software.

RESULTS

Plants have a long history of use in the treatment of cancer. It has become a necessity to understand the folk medicine which laid down on complementary and alternative medicine research that deals with cancer management. Phytochemistry showed the presence of flavonoids in the ethyl acetate crude leaf extract (Table 1). TLC using ethyl acetate, methanol and water (100:16.5:13.5) and (70:15:5) as solvent system showed eight and seven bands respectively on TLC plate in UV and ammonia brown, yellow and fluorescent green bands confirmed the presence of flavonoids with $R_F \times 100$ - 90.9, 83.9, 83.2, 18.2, 14.7, 11.9, 8.4, 7.0 and 82.2, 80.5, 78.9, 74.4, 71.1, 61.1, 56.7 in first solvent and second respectively (Figure 1).

Acute oral toxicity

LD 50 determination indicates safety profile of the drug. From the acute oral toxicity study it was observed that oral administration of extract at 2000 mg/kg did not cause mortality in animals. The extract was found safe in animals dosed at 2000 mg/kg. ED₅₀ for extract was 200 mg/kg.

Sub acute toxicity studies

Histological examinations showed that there was no significant variation visualized in histology of vehicle treated and test sample treated animals. Normal architecture of kidney was found to be preserved with normal glomeruli. Epithelial linings were normal. No significant effect on connective tissue. Distal convoluted tubule, proximal convoluted tubule and their brush border were also in good condition. Also there was no significant variation was seen in histology of liver of vehicle treated and ethyl acetate crude extracts treated animals. Normal architecture of liver was found to be preserved. Basic hepatic lobule, portal triad, sinusoids, hepatocytes were found to be normal in both treatment groups. Vascular lining of central veins was also found to be normal. No sign of inflammation or necrosis was present (Figure 2).

Biochemical examinations

The results of biochemical estimations are reported as mean $\pm$ SD of six animals in each group (Table 1). The parameters showed the safety profile of the extract.

Antitumor Study

Results of antitumor activity study of ethyl acetate crude leaf extract of *Parkinsonia aculeata* L. showed that extract exhibited significant antitumor activity. The animals were given drug per oral (p.o.) for test sample and cancer cells were injected subcutaneously (s. c.) from cell suspensions. Palpable as well as measurable tumors were observed in vehicle and treatment groups were seen. Treatments with ethyl acetate extract p.o. D₁ at 100 mg/kg bwt. and D₂ at 300 mg/kg bwt. for 14 days reduced the tumor growth in a dose dependent manner in BALB/c mice inoculated with B16F10 tumor cells. Status of antioxidant enzymes in vehicle, D₁ and D₂ treated mice showed that the extract possesses antioxidant

potential which may be one of the mechanism of action in antitumor action of extract (Table 3).

DISCUSSION

Melanoma is the deadliest form of skin cancer and any practical solution in combating this type of cancer is of paramount importance to public health. Because of various side effects of the allopathic drugs it is worth evaluating a plant based therapy which is not a systemized study. Crude extracts from plants like *Colubrine marocarpa*, *Hemiangium excelsum* and *Acacia pennatula* have been shown to possess a selective cytotoxic activity against human tumor cells KB, HCT-15 COLADCAR and UISOSQC-1¹⁹. Another member of the family leguminosae has been shown to have significant anti-breast cancer potential *P. zeylanica* L. extract is cytotoxic to tumor cells²⁰. In the Palestinian and Israeli territories, extracts of *Teucrium* and *Pistacia lentiscus* are known to treat cancer. Such extracts are rich in flavonoids²¹. Similar results also reported by^{22,23}. Our previous studies showed that flavonoid fraction from *P. aculeata* L. leaves extract possess cytotoxic activity *in vitro* against B16F10 mice melanoma cell lines²⁴. This purified fraction on further chromatographic and spectral analysis revealed the presence of apigenin in purified fraction which is an important flavonoid as it possesses antioxidant and antitumor potential. The TLC synopsis showed that the yellow or invisible bands were visualized as more intense and yellow colored in ammonia confirms the presence of flavonoids which visualized as a brown colored band in UV which again confirmed the presence of flavonoids. The green fluorescent band is characteristic of apigenin C-glycosides e.g. vitexin, isovitexin. The yellow zones again are characteristic of flavones C-glycosides like iso-orientin. The presence of C-glycosides was also confirmed by phytochemistry. These components may be apigenin, vitexin and isovitexin glycosides. Similar results also reported by Elegami *et al*²⁵. Apigenin's preventive effect is shown to be mediated through induction of p53 expression, which causes cell cycle arrest and apoptosis^{26, 27}. Similar results also reported by Fotsis *et al.*; Stefani *et al*^{28, 29}. Our previous study also reveals that the extract possesses potent antimicrobial activity against some bacteria causing Urinary Tract Infection in Humans³⁰. Further the extract can be stored at room temperature without any loss of activity and degradation for longer periods. This may be a plus point in drug development. The crude extract possesses *in vitro* antioxidant potential with 75.5 % Inhibition and IC₅₀ value of 0.38 mg/ml³¹.

CONCLUSION

The ethyl acetate crude leaf extract contain the active principles that mediate the anticancer property. The active components that mediate the biological property may be majorly flavonoids viz. apigenin, vitexin, iso-vitexin, orientin, iso-orientin and chrysoeriol. Further work is needed to ascertain the mechanism of actions of these flavonoids. The study also provides the safety profile and dosage of the ethyl acetate crude leaf extract. Thus the plant's leaves could be good source of natural and potent chemotherapeutic. The results showed that the ethyl acetate crude extract of *P. aculeata* L. leaves possess antitumor potential and is a good source of various flavonoids. It should be considered for further analysis for complete structure determination and pharmacognosy studies. It will provide a novel natural drug for melanoma and various ailments. It could be a promising alternative to synthetic drugs.

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