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ANTICANCER EFFECT ON ORANGE PEEL EXTRACT IN MELANOMA TUMOUR INDUCED C57BL HYBRID MICE MODEL

K. BALAMURUGAN [*].

[*] Assistant Professor, Department of Pharmacy, FEAT, Annamalai University, Annamalai Nagar, Chidambaram - 608002, Tamil Nadu, India. E-mail: placementbala@yahoo.co.in

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Abstract

Melanoma is a highly aggressive cancer that exhibits metastasis to various critical organs. Unlike any other cancer cells, melanoma cells can synthesize melanin in large amounts, becoming heavily pigmented. Until now the role of melanin in melanoma, particularly the effect of melanin presence on the abilities of melanoma cells to spread and metastasize remains unknown. The employment of animal models to test therapeutic strategies against melanoma growth and metastatic spread is of key relevance for cancer biologists. The current research was focused to studythe anticancer effect on orange peel extract in melanoma tumour induced c57bl hybrid mice model. Significant reduction of tumour growth was found treated with Orange peel which was subcutaneously injected with B6F10 melanoma. Survival time was also increased in compare with control untreated tumour bearing mice. The orange peel hydromethanolic extract of (200mg/kg, 400mg/kg and 600mg/kg) drug dosing showed decrease in the micronucleus formation respectively.

Key Words: Orange peel, Melanoma, c57bl hybrid mice model**.**

Introduction:

Melanoma is the most invasive skin cancer with the highest risk of death. Prevention and early treatment are critical, especially if you have fair skin, blonde or red hair and blue eyes. About 30% of melanomas begin in existing moles, but the rest start in normal skin. The risk of melanoma seems to be increasing in people under 40, especially women. (1) Knowing the warning signs of skin cancer can help ensure that cancerous changes are detected and treated before the cancer has spread. Melanoma can be treated successfully if it is detected early.Malignant melanoma is an extremely rare malignancy in the Indian subcontinent and South-east Asia. Two FDA-approved agents, Ipilimumab and Pembrolizumab, target immunomodulatory proteins are available to cure melanoma.(2,3)Despite recent advances in

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melanoma therapeutics, prognosis remains poor, with a five-year survival rate of 16% for patients with distant metastases. Even though the immune checkpoint inhibitors well-tolerated, their use is associated with several adverse effects including fatigue, nausea, vomiting, diarrhoea, and dermatological reactions.(4) Hence, the current research was focused to study the anticancer effect on orange peel extract in melanoma tumour induced c57bl hybrid mice model.

Citrus sinensis(orange) has been used traditionally to treat ailments like constipation, cramps, colic, diarrhea, bronchitis, tuberculosis, cough, cold, obesity, menstrual disorder, angina, hypertension, anxiety, depression and stress. (5) It belongs to *Rutaceae* family it is a citrus fruit and a hybrid of pomelo and mandarin orange. *Citrus sinensis* is a small evergreen tree originally domesticated in subtropical Asia, it is cultivated in Telangana, Andhra Pradesh, Maharashtra, Madhya Pradesh, Karnataka, Punjab, Bihar, Assam, Mizoram and Jammu & Kashmir. (6)

Preparation of orange peel extract

The fresh orange fruits were collected from local shops of Bhopal (M.P), India. Freshly collected orange peels were rinsed in double distilled water and dried in 40˚ C in hot air oven for 60 h and then powdered with mechanical grinder. The powder was passed through sieve No.40 and stored in air-tight container. The powder was extracted with in solvent methanol and hydro-methanol (50 % v/v) by hot continuous percolation method in Soxhlet apparatus for 10 hrs individually. The extract was filtered and concentrated in water bath under reduced pressure to obtain semisolid material which was then powdered. (7)

Phytochemical Screening

Preliminary phyto - chemical screening was carried out for methanol and hydro-methanol orange peel extract by following standard procedure. The test to identify carbohydrates and reducing sugars, glycosides, alkaloids, phenolic compounds, tannins, phytosterols,proteins, saponinsand flavanoids were carried out. (8) The results are given in **Table: 1**

Melanoma Assay

C57BL hybrid mice having weight of 25 - 30 gm were kept in quarantine for 10 days under standard husbandry conditions (27˚C, RH 65 ±2 %) for 12 h in dark and light cycle respectively and were given standard food and water *ad libitum.*(9) All the experiments were performed as per the CPCSEA norms after obtained the approval of the IAEC, JNCH & RC Bhopal dated 17.05.2011.

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In vivo melanoma models have typically relied on the growth of tumour xenografts in immune compromised mice. Several genetically engineered mouse models have now been developed which allow the generation of spontaneous melanoma. The melanoma cell line was obtained from National cell science centre .pune and maintained in our laboratory, C57BL mice of both sexes of the mean weight of 25gm and 6-7 weeks were obtained from the animal's colony of out institutes .all the mice were kept at controlled light and temperature condition. Cell suspension having about 5 lakhs cells were injected to every mouse by subcutaneous injection in 200 µL of phosphate-buffered saline (PBS), using 28 gauge needles (10)

Method

Mice were divided in to 6 groups of six in each, Group I animals were treated with O.P. E 200mg/kg + Cyclophosphamide 50mg/kg; Group II animals were treated with O.P. E 400mg/kg + Cyclophosphamide 50mg/kg ;Group III animals were treated with O.P. E 600mg/kg + Cyclophosphamide; 50mg/kg Group IV animals were treated with O.P. E 200mg/kg alone and Group V animals were treated with Cyclophosphamide 50mg/kg alone. The mice were shaved with the help of hair removal cream. Male donor mice for were euthanized at 8–10 weeks of age and sacrificed. The bone marrow was flushed from femurs and tibias bearing the melanoma tumour in saline and properly chopped then filtered. The cells were counted with the help of Neuber chamber and 0.5 ml of the cells were transferred and assumed to consist of 5 lakhs cells. After the arrival of tumour the dosing was started and the effect of drug in inhibition of the growth of cancer was seen and the dosing was done for alternate days and the height, length, breadth was measured everyday and it was continued for a month.

Animals were sacrificed and bone marrow was extracted, spread on slides and the frequency of micro nucleated PCE cells was scored and compared among treatment groups. Cells which stain uniformly positive for RNA are referred to as polychromatic of polychromatophilic erythrocytes (PCEs) cells which do not stain positively for RNA are referred to as homochromatic erythrocytes (NCEs) an increase in frequency of micro nucleated PCEs relative to the vehicle control group indicates the test substances induced chromosomal damage or lagging chromosome in the nucleated erythrocytes cells.(11)

Preparation of the smear:-

The tube was centrifuged at 1000rpm for 5 min. The supernatant is removed the cells in the sediment are carefully mixed by aspiration and a small drop of viscous suspension was put on the end of slide and spread by pulling the material behind a polished cover glass held at an angle of 45 degree. (12) The preparation was then dried and fixed for 2-5 min . Staining was carried out in ordinary vertical staining jars according to the following procedure. Stain for 5 min May –

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Gruenwald solution and stain for 10 min in Giemsa then slides rinsed in distilled water , blotted , clean back side of slides with filter paper then dry the slides on slide warmer .(13)

Analysis of Slides:-

PCEs were scored for micronuclei under the microscope at least 1000 PCEs per animals were scored for the incidence of micronuclei. The ratio of PCEs to NCEs was determined for each animal by counting a total 1000 erythrocytes. The following parameters like Micronucleus counting, PCE/ NCE ratio and % reduction in mutation were analysed.(14) The photograph of showing micronucleus in PCE cell are shown in Fig 1.

RESULTS

TABLE: 1 PHYTOCHEMICALS PRESENT IN THE METHANOLIC EXTRACT OR HYDROMETHANOLIC EXTRACT OF ORANGE PEEL

S.no No	Phytochemical test	hydromethanolic extract	methanolic extract
1.	**Test for carbohydrates and reducing sugars**		
A.	Fehlings solution	+	+
B.	Benedict test	+	+
II	**Test for glycosides**		
A	Legals test	+	+
B	Borntragers test	+	+
III	**Test for alkaloids**		
A	Dragendroffs test	+	+
B	Mayers test	+	+
IV	**Test for phenolic compounds**		
A	Ferric chloride test	+	+
V	**Test for tannins**		
A	Acetic acid test	+	+
VI	**Test for phytosterols**		
B	Salkowski test	-	-
VII	**Test for proteins**		
A	Biuret test	-	-
VIII	**Test for saponins**		
A	Foam test	+	+
B	Sodium bicarbonate test	+	+
IX	**Test for Flavonoid**		
A	Sodium hydroxide test	+	+
B	Lead acetate test	+	+

"+" present "- "absent

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TABLE: 2 TABLE SHOWING EXPERIMENTAL PROTOCOL OF MICRONUCLEOUS ASSAY

Sl.No	Treatment	No of animals
1	Group I animals were treated with O.P. E 200mg/kg + Cyclophosphamide 50mg/kg	4
2	Group II animals were treated with O.P. E 400mg/kg + Cyclophosphamide 50mg/kg	4
3	Group III animals were treated with O.P. E 600mg/kg + Cyclophosphamide 50mg/kg	4
4	Group IV animals were treated with O.P. E 200mg/kg alone	4
5	Group V animals were treated with Cyclophosphamide 50mg/kg alone	4

TABLE: 3 ANTICANCEROUS ACTIVITY OF HYDROMETHANOLIC EXTRAXT OF ORANGE PEEL ON HYBRID MICE

s.no	Group	Tumour volume	Survival days	Increases life span (%)	Inhibition rate (%)
1	Animals were treated with Orange peel extract	1346.68	20	-	46.07
2	Animals were treated with O.P. E 200mg/kg + Cyclophosphamide	1093.79	28	40%	59.2
3	Animals were treated Cyclophosphamide	531.58	12	-	78.7
4	Animals were Untreated	2437.25	20	-	

Control vs orange peel extract Cyclophosphamide P>0.05

Table: 4 – EFFECT OF ORANGE PEEL ECTRACT ON MICRONUCLEUS FORMATION IN MOUSE BONE MARROW CELL

SL.NO	TREATMENT	MNPCE ±S.E	PCE/NCE RATIO
1	Group I animals were treated with O.P. E 200mg/kg + Cyclophosphamide 50mg/kg	1±0.366	0.535±0.00036
2	Group II animals were treated with O.P. E 400mg/kg + Cyclophosphamide 50mg/kg	1±0.408	0.423±0.077
3	Group III animals were treated with O.P. E 600mg/kg + Cyclophosphamide 50mg/kg	0.334±0.211	0.925±0.053
4	Group IV animals were treated with O.P. E 200mg/kg alone	0.333±0.211	0.8775±0.035
5	Group V animals were treated with Cyclophosphamide 50mg/kg alone	3.167±0.00036	0.679±0.00072

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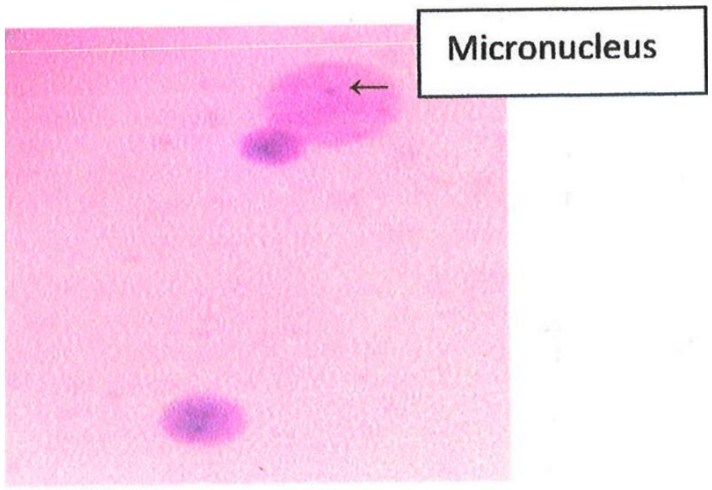

Figure : 1 Photograph Showing Micronucleus in PCE cell

The phytochemical screening of the orange peel showed that phytoconstituents such as flavanoids, alkaloids, glycosides, tannins, phenolic compounds are present in the hydromethanolic and methanlic extract of *Citrus sinensis* (orange peel) but absence of steroids and proteins was observed in the extract.

The micronucleus study showed that the single application of the orange peel extract at the dose of 200,400,600mg/kg body wt prior to the administration of Cyclophosphamide have significantly prevented the micronucleus formation in the dose dependent manner. The PCE/NCE ratio of orange peel extract alone not suppressed as compared to control group. In this group of mice treated with Orange peel showed significant reduction in tumor size and their day of survival was also increased as compared with control group.

Therefore, significant reduction of tumor growth was found treated with Orange peel which was subcutaneously injected with B6F10 melanoma. Survival time was also increased in compare with control untreated tumour bearing mice. The micronucleus formation determines the genotoxic effects of the drug. The anti- mutagenecity of the drug could be evaluated from the result itself as the Cyclophosphamide which is an anticancer drug is also an mutagenic agent. Thus, the comparative study between different drug doses and Cyclophosphamide was done that results evaluated that the number of micronucleus formation was higher in Cyclophosphamide (50 mg/kg.). The orange peel hydromethanolic extract of (200mg/kg, 400mg/kg and 600mg/kg) drug dosing showed decrease in the micronucleus formation respectively. Thus it could be concluded that when the concentration of drug is increased then the antimutagenic activity is maximum.

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Conclusion:

In summary, the orange peel hydromethanolic extract of (200mg/kg, 400mg/kg and 600mg/kg) drug dosing showed decrease in the micronucleus formation respectively in melanoma tumor induced C57BL hybrid mice model. Further studies are needed to study in higher animals and the mechanism of action behind it.

Conflicts of interest: None declared.

Ethical approval: IAEC, JNCH & RC Bhopal dated 17.05.2011.

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