IN VIVO ANTICANCER ACTIVITY OF THE LEAVES AND FRUITS OF *MORINGA OLEIFERA* ON MOUSE MELANOMA

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Summary

In vivo study for exploring the *anticancer* activity of leaves and fruits of *Moringa oleifera* were explored on B16 F10 melanoma tumor. Tumors (100±10 mm^3) were grown intradermally in hybrid (C57BL X Swiss albino) mice of either sex. The mice were treated orally with hydromethanolic (HMF1, HML1) and methanolic extract (MF2, ML2) of fruits and leaves respectively at 500 mg/kg b.wt. and further ML2 at 1g/kg b.wt. for 15 days. Tumor growth kinetics and survival were studied using volume doubling time (VDT), growth delay (GD), mean survival time (MST), percentage increase in life span (% ILS) values. All the treatments produced a significant increase in VDT and GD. At 500 mg/kg, MF2 produced higher VDT and GD than ML2. MST showed significant increase only in ML2 at both doses, while %ILS values were significant in the HML1 and ML2. Being as dietary plant, daily intake of fruits and leaves of this plant can delay the tumor growth and increase the life span of the cancer patients.

Key words: *Moringa* oleifera; Anticancer Activity; B16F10 Melanoma; Volume Doubling Time; Growth Delay; Mean Survival Time, Percentage Increase In Life Span; Tumor Regression; Tumor Survival.

Introduction

Drumstick/Malunggay (*Moringa oleifera, Family: Moringaceae)* [1] a native of Indian medicinal plant has been reported as the significant sources of vitamins (A, B, C, E, riboflavin, nicotinic acid, folic acid, pyridoxine, ascorbic acid, beta carotene), iron, calcium and alpha tocopherol [2]. Anti tumor promoting activity of the leaves and pods of drumstick has also been reported [3]. Additionally it is an important source of glucosinolate precursors of the **isothiocynate** group of chemopreventives that can inhibit carcinogenesis [4]. It contains the phytochemical **niaziminin**, which is found to have molecular components that can prevent the development of cancer [5]. **Niazimicin**, a compound isolated from *M. oleifera* have also been reported to have potent anti tumor promoting activity in two stage carcionogenesis in mouse skin using 7,12-dimethylbenz (a) anthracene [DMBA] as an initiator and 12-O-tetradecanoyl-phorbol-13-acetate [TPA] as a tumor promoter [6].

Ethanolic extract of Leaves of this plant have found to reported various constituent like ***Thiocarbamate like Niazinin A,Niazinin B, Niazimicin, Niaziminin A, Niaziminin B,*** **Carbamates like Niazimin A, Niazimin B, Niazicin A, Niazicin B, Two nitrile glycosides like Niazirin, Niazirinin** and three mustard oil glycosides like 4- [[4'- O-acetyl –4-(α-L-rhamnosyloxy) Benzyl] isothiocynate, **Niaziminin-A, Niaziminin-B, Niazirinin-A, Niazirinin –B** [5,7-10].Quercetin and various phytocostituents responsible for anticancer activity have also been reported in this plant [11-13]. Moringa leaves are found to be a potential source of antioxidants [14]. Leaves of *M. oleifera* were a rich source of kaemferol [15]. Nicotinic acid which has also been reported some of activity against metastasis for B16F10 melanoma cell lines [16], is also reported in this plant [17]. Consumption of natural antioxidants from foodstuffs can protect the human body from the attack of free radicals [18]. Studies on human epidermoid carcinoma of nasopharynx using 50% ethanolic extract of *M. oleifera*, have also confirmed its role in cancer inhibition [19]. In cell culture studies the ethanol /water extracts of different parts reported inactive using Leuk P388, Leuk P1210, Sarcoma180, ASC [19] and CA-9KB cell lines [19,20,21]. Further, the antioxidant and free radical scavenging activities of methanolic (80%) and ethanolic (70%) extracts of *M.oleifera* suggests its utility in treatment of the cancer/tumor inhibition [14]. So it might be possible that these antioxidants may provide preventive /therapeutic response in management of the cancer. Furthermore *In vivo* radio protective activity using 50% methanolic extracts of leaves has also been reported [22].

The objective of the undergoing work was to investigate the cancer therapy potential of *M.oleifera*, which could circumvent the limitations of chemotherapy and radiotherapy for the treatment of cancer. Therefore the envisaged work was designed to study the *in vivo* anticancer effect of the leaves and fruits of drumstick plant on solid transplantable B16 F10 murine melanoma tumor.

Materials and Methods

Drug: Fruits and leaves of plant were collected locally, washed and shade dried. The crude hydromethanolic and metahnolic extracts of fruits and leaves were prepared using 75% and pure 100% methanol as menstruum respectively by cold maceration method [23-26]. The hydrometahanolic extracts of fruits and leaves were coded as HMF1 and HML1 Similarly the pure methanolic extracts of fruits and leaves were coded as MF2 and ML2. The extracts were dried and dissolved in double distilled water to get required concentration.

Tumor: The B16F10 mouse melanoma procured from Jawaharlal Nehru Cancer Hospital and Research Center Bhopal propagated by serial transplantation in adult hybrid (C57BL X Swiss albino) mice. Solid tumors were produced by intradermal injection of $5X10^5$ viable cells into the hybrid mice of either sex (25±5gms).

Experimental Design: Once the palpable tumor developed, its diameter in three perpendicular planes (D1, D2, D3) were measured using a vernier caliper and tumor volume (V) was calculated using the formula:

$$\mathbf{V = \pi/6 * D1 * D2 * D3}$$

Treatment: Treatments were commenced when the tumor size reached to the (100±10 mm^3). (Table I) The control received an equal amount of double distill water (DDW). HMF1, HML1, MF2 and ML2 were given orally at the dose of 500 mg/kg b.wt. for 15 days. Based on the preliminary results, ML2 was given orally at the dose of 1g/kg b.wt. for 15 days(Table –I)

Table I: Treatment protocol of leaf and fruit extracts of *Moringa oleifera* on mice bearing melanoma.

S.No.	Group	Number of Animals	Treatments	Dose (mg/kg)	Route	Duration (Days)
1	Control	6	Double distill water	…	Oral	15
2	Group I HML1	7	75% methanolic leaf extract	500	Oral	15
3	Group II ML2	8	100% methanolic leaf extract	500	Oral	15
4	Group III HMF1	12	75% methanolic leaf extract	500	Oral	15
5	Group IV MF2	6	100% methanolic fruit extract	500	Oral	15
6	Group V ML2	7	100% methanolic leaf extract	1000	Oral	15

The tumor response was assessed by the tumor growth kinetics:

1. Tumor Regression Studies. [27, 28]

Volume Doubling Time: Time required for the tumor to attain double the treatment volume.
Growth Delay: The difference in time, in days between the treated and untreated tumors to reach five times of the treatment volume.

Complete Response (CR): Complete regression with no regrowth at the primary site during 120 days of observation.

Partial Response (PR): more than 50% regression in tumor size.
No response: less than 50% regression in tumor size.

2. Survival Studies [29, 30]: Tumor free animal survival time was recorded up to 120 days. Mean survival time and median survival time (MST) was also calculated. The tumor response was assessed on the basis of the **percentage increase in life span (%ILS)** [29, 30].

$$\%ILS: \quad \frac{\text{Median survival time of (treated group – control group)}}{\text{Median survival time of control group}}$$

An enhancement of life by 25% or more over that of control was considered as effective antitumor response [31].

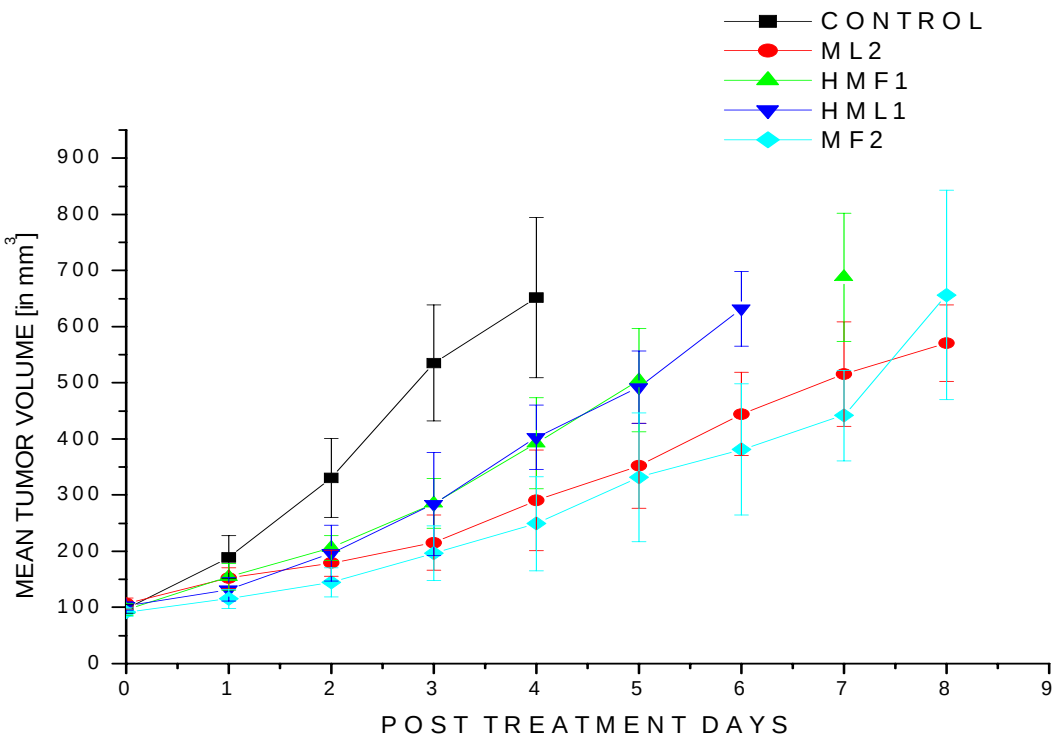
Statistical Analysis was done by one-way ANOVA using microcal origin version 6.0, Graph Pad In Stat (GPIS) statistical software, U.S.A and Kyplot. All the values were expressed as Mean ±SE. The data of the volume doubling time and mean survival time were statistically analyzed by Student 't'test and data of growth delay were analyzed by one-way ANOVA using microcal origin version 6.0, Graph Pad In Stat (GPIS) statistical software, U.S.A and Kyplot. $p<0.05$ was considered to be significant.

Results

In vivo antitumor activity of the hydromethanolic and methanolic extracts (by using 75% and 100% methanol as solvent) fruits and leaves of *M.oleifera* were studied on experimental B16F10 mouse melanoma. The different fruit and leaf extracts (HMF1, HML1, MF2, and ML2) were orally administered to tumor bearing mice with tumor volume (100±10 mm^3) at dose of 500 mg/kg for 15 days. The tumor growth kinetics and survival studies of the different treatment groups were evaluated with VDT, GD, MST, and %ILS.

Tumor growth kinetics demonstrated that none of the treatment produced any complete response or any partial response at 500 mg/kg dose. In all treatments only delayed growth of tumors shown by an increase in VDT and GD were observed, but the tumor growth inhibitory effect was not sufficient to cure the tumors(Table II). Among the various treatments, the maximum regression in terms of VDT and GD were observed in case of the MF2 only and the tumor volume was observed one forth $(1/4)^{th}$ of control group and VDT and GD were observed 3 and 4 times respectively of the control group. During the treatment, the maximum VDT and GD were observed with MF2 having ($p<0.001$) which was less than 5 days (Figure I).

Figure I: Effect of leaf and fruit extracts on the regression of tumor growth.



The response of VDT and GD of ML2 was also significantly higher with ($p<0.001$) as compared to the control, but the response was inferior to that of MF2. The curative response of HMF1 and HML1 was almost similar in terms of VDT and GD (Table II).

Table II: Effect of leaf and fruit extracts of *Moringa oleifera* on mice bearing melanoma.

S.No.	Treatment Groups	Number of Animals	VDT (Days) [Mean ±SE]	GD (Days) [(Mean ±SE)]
1	Control (DDW)	6	1.146±0.4365	0
2	Group I (HML1) 500mg/kg	7	2.15±0.263[a]	2.054±0.21[a]
3	Group II (ML2) 500mg/kg	8	2.781±0.2895[c,e]	3.82±0.294[c,d,e]
4	Group III (HMF1) 500mg/kg	12	1.9±0.122[a]	2.09±0.245[b]
5	Group IV (MF2) 500 mg/kg	6	3.420±0.50[c,d,e]	4.05±0.424[c,d,e]
6	Group V (ML2) 1000 mg/kg	7	3.197±0.270[c,d,f]	3.026±0.339[c]

[a] :p <0.05, [b] :p <0.01, [c]:p <0.001 compared to control; [d] :p <0.05 compared to HML1 ; [e] :p <0.01 compared to HMF1; p<0.001 compared to HMF1.

The long-term Survival studies demonstrated that none of the treatment resulted in free survival of animals up to 120 days. All the leaf treatments (HML1, ML2) showed effective survival of animals. The fifty percent animals were survived up to maximum 37 -days in ML2, which was 14 days more than control group (23 day). The 50% survival was significantly higher in HML1, ML2 but the HMF1 and MF2 showed inferior results with survival studies.

Significantly higher MST was observed only with ML1, ML2 [33.28, 36.88days respectively with (p<0.05) (p<0.01) respectively]. The ML2 was most effective in increasing MST (p<0.01).

None of the extract produced any extremely significant increase in MST, but a prolonged life span was observed with HML1 and ML2. Oral administration of ML2 at 500mg/kg dose produced 48% increase in life span, which was highly significant in all treatments **(Table III).** The percentage increase in life span was 32%, 12% and 22% for HML1, HMF1 and MF2 respectively (Table III).

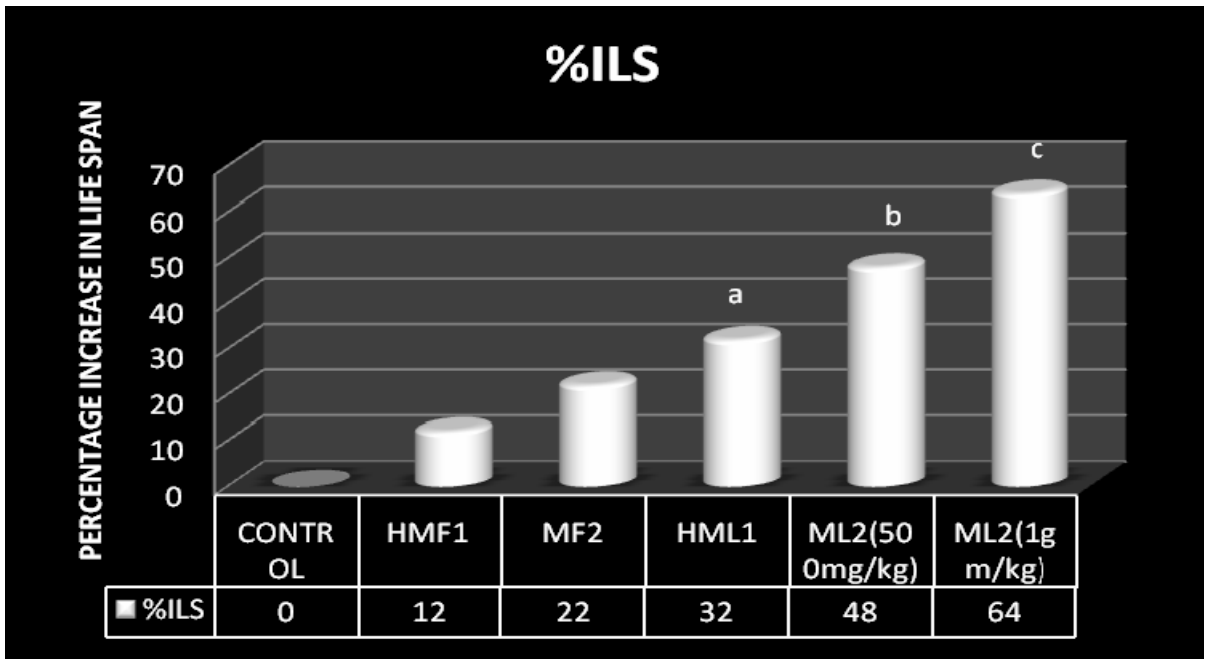
Table III : Long-term survival studies of mice bearing Melanoma tumor after treatment with leaf and fruit extracts of *Moringa oleifera.*

S.No.	Treatment Groups	Number of Animals	Mean Survival Time [Days (Mean ±SE)]	Median Survival Time [days ((Mean ±SE)]
1	Control	9	25.33±2.84	25±2.84
2	Group I (HML1) 500 mg/kg	7	33.285±1.37 [a]	33±1.37
3	Group II (ML2) 500 mg/kg	9	36.88±1.019 [b, d, e, f]	37±1.019
4	Group III (HMF1) 500mg/kg	6	30.5±2.53	28±2.53
5	Group IV (MF2) 500 mg/kg	6	32.33±2.12 [c]	30.5±2.12
6	Group V (ML2) 1000 mg/kg	7	39.42±2.55 [b, e, f, g]	41±2.55

[a] :p <0.05, [b] :p <0.01, [c] : marginally significant, compared to control; [d] :p <0.05 compared to HML1; [e] :p <0.05 compared to HMF1 ; [f]: (Marginally significant) compared to MF2; [g]: (Marginally significant) compared to HML1.

An increase in life span >25% is considered to as effective antitumor response as discussed earlier [32]. HMF1 and MF2 showed inferior/ineffective results in increasing the MST and life span (Figure II).

Figure II: Effect of leaf and fruit extracts on survival (% ILS) of mice bearing melanoma.



>25% is considered to be having effective anticancer response.

a: Significant

b: very Significant

c: Extremely Significant

The regression studies revealed that MF2 and ML2 was effective in increasing the VDT and GD, while the survival studies showed ML2 was effective in increasing the survival and life span. So based on the observations recorded above it has been thought to study the effect of ML2 at the higher dose (1gm/kg), there was no CR or PR observed. Tumor regression studies with the higher dose revealed that the growth pattern of the tumors was similar as that of the 500mg/kg, but the growth rate was found less than MF2 with 500mg/kg. In case of VDT, the response of ML2 (1gm/kg) was still inferior to MF2 but found higher than ML2 (500 mg/kg) and the response towards GD was found lower as compared to the ML2 and MF2 (500 mg/kg). The VDT and GD were 3 times of the control group. The following order of the response of VDT is observed: **MF2> ML2 (1gm/kg)> ML2 (500mg/kg)**. Where as the order of the response of GD: **MF2> ML2 (500mg/kg) > ML2 (1gm/kg)**.

Survival studies demonstrated that higher dose showed effective survival of animals and the animals were survived up to 46 days, which was11 days more than control group. With the higher dose of ML2, the percentage survival was 85.71 % on the 32nd day as compared to the control (55.55%). The fifty percent animals survived up to maximum 42 -days that was 19 days more than control group (23 day). Higher 50% survival was observed in ML2 with higher dose. MST was highest ($p<0.01$) with this dose. The %ILS was higher (64%) with ML2 (1gm/kg) than ML2 (500mg/kg) (48%), which was highly significant in all treatment groups **(Table IV).**

Table IV:Effect of leaf and fruit extracts of *Moringa oleifera on* Percentage increase in life span (%ILS) of mice bearing Melanoma tumor.

S.No.	Treatment Groups	Number of Animals	Percentage increase in life span
1	Control	9	0
2	Group I (HML1) 500mg/kg	7	32
3	Group II (ML2) 500mg/kg	9	48
4	Group III (HMF1) 500mg/kg	6	12
5	Group IV (MF2) 500 mg/kg	6	22
6	Group V (ML2) 1000 mg/kg	7	64

>25% is considered to be having effective antitumor response.

Discussions

A majority of the carcinogenic agents are regarded as powerful generators of free radicals leading to cancer. Natural compounds have demonstrated strongest antioxidant and anticancer activity with multifunctional activity which also binds to and modulates activity of protein kinase involved in signal transduction cascades, show cytotoxic and cytostatic activity towards cancer cells [32]. In present study *in vivo* anticancer activity of hydromethanolic and methanolic extracts of fruits and leaves were tested on experimental B16F10 mouse melanoma.75% methanol and 100% methanol were taken for the extraction of the leaves and fruits.

The growth rate of the tumors in control group follow a sigmoidal growth pattern, but with the other treatments, MF2 extract treated tumors was showing lower growth rate which gives an effective regression on tumors. ML2 and MF2 with the 500 mg/kg dose were found most potent towards tumor regression /tumor inhibitory property in terms of increased VDT and delayed growth (GD) and only ML2 was potent in increasing the survival and life span. But ML2 and MF2 were not having as much potential for tumor cure. Only better tumor inhibitory response was observed with these two treatments. But survival studies concluded that ML2 was effective in long-term survival. Over all a secondary tumor inhibitory response (than MF2) and better /effective survival was only observed in ML2 with 500 mg/kg dose.

Further in order to evaluate the dose dependent changes in tumor regression and survival parameters /studies, the dose of the ML2 was increased up to 1000mg/kg (LD50) [20,33,34]. With the higher dose of ML2 (1000mg/kg), the regression effect in terms of the increased VDT reflects effectiveness of methanolic leaf extract on the early tumors /early stages of tumors i.e. on the actively dividing cells. Further an insignificant difference in GD via ML2 at 500mg/kg and at 1000mg/kg doses possibly suggests ineffectiveness of higher dose on advanced tumors/advanced stages of tumors/later stages.

The possible anticancer effect of methanolic (100%) extracts of leaves and fruits of this plant could be due to presence of number of phytoconstituents including Niazimicin [7,8,9], Niaziminin [7,8,9], Quercetin [11,12,13] Nicotinic acid [17], various vitamins and antioxidant combinations making a synergistic effect leading to better tumor regression /antitumor effect.

Fruit and leaves are having a number of different phytoconstituents, which reflects that a particular single constituent may not be responsible for antitumor effect, but this effect was resulted from combined effect or cumulative/synergistic effect of its phytoconstituents. Thus results show that low nontoxic doses of an extract of leaves and fruits have a good antitumor effect on mouse melanoma tumor and leaf extract also enhances the survival time and life span of the cancer patients.

A dose of 500mg/kg (about 1/15 th of its LD_{50}) [22] of leaf extract appears to be optimum /effective to obtain/ to evoke a good antitumor response with out any noticeable toxicity. Further LD_{50} of fruit extract [34] did not promote to attempt this study at higher dose.

Conclusion

The regression studies reveal that fruit and leaves were effective in delaying the tumor growth while survival studies reveal that leaves were the most effective in increasing the survival time .So daily intake of leaves and fruits of this plant in diet can delay the tumor growth and increase the life span of the cancer patients. Further this plant can be used as a protective measure in cancer patients undergoing radiation therapy as this plant has been demonstrated *in vivo* radio protective activity [23]. As moringa leaves and fruits are used as a dietary vegetable and is freely available in our country, it's worthwhile to conduct detailed studies in order to explore the full potential of this plant in the management of the cancer. These findings indicate a possible tumor therapy potential of this plant, as this plant reflects higher antioxidant activity as compared to the Vitamin E, it could be a cancer preventive effect.

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