Aegle marmelos (L.) CORREA Inhibits the Proliferation of Transplanted Ehrlich Ascites Carcinoma in Mice

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The anticancer effect of hydroalcoholic extract of Aegle marmelos (AME) was studied in the Ehrlich ascites carcinoma bearing Swiss albino mice. The spatial effect of various AME administration schedules showed that six-day administration increased the survival of tumor bearing mice. The best antineoplastic action of AME was obtained when AME administered through intraperitoneal route than the oral route at equimolar dose. Administration of AME once daily for six consecutive days to the tumor bearing mice caused a dose dependent remission of the tumor at 400 mg/kg body weight, where the greatest antitumor effect was observed and the higher doses showed toxic manifestations. A 24-d lengthening in life span was observed in EAC animals treated with 400 mg/kg AME. This dose of 400 mg/kg was considered as the best dose, where the animals survived up to 43 d post-tumor-cell inoculation as against no survivors in the saline treated control group. The antitumor activity when tested for different schedules for triple administrations, the best effect was observed for 1–2–3, followed by 1–3–5 and 1–5–9 d, respectively. Stage specific evaluation of AME inhibited the increase in body weight gain in animals due to tumor development during early stages only. The AME treatment resulted in a dose dependent elevation in the median survival time (MST) and average survival time (AST) up to 400 mg/kg AME and decline thereafter. The effective dose of 400 mg of AME is 1/6th of the LD50 dose, which increased the MST and AST up to 29 and 27 d, respectively. The acute toxicity study of AME showed that the drug was non-toxic up to a dose of 1750 mg/kg b. wt. The LD50 and LD10 was found to be 2000 and 2250 mg/kg.

Key words Aegle marmelos; anticancer; Ehrlich ascites carcinoma; tumor; mean survival time; average survival time

Plants like Catharanthus roseus, Podophyllum peltatum, Podophyllum emodi, Taxus brevifolia, Ochrosia elliptica and Camptotheca acuminata, have provided active principles, used to control advanced stages of several malignancies in clinical settings. Most of these chemotherapeutic agents have provided active principles, Ehrlich Ascites Carcinoma in Mice may be counteracted by another component, which may not be effective at non-toxic dose levels. Further many of the potent antineoplastic drugs are highly expensive, mutagenic, carcinogenic and teratogenic. Therefore, the current investigations should be directed to find out alternative drugs, which are highly effective at non-toxic doses, inexpensive and accessible to the common man. This can be achieved by screening newer molecules or plant products, which may be effective at non-toxic dose levels.

Ayurvedic system (Indian system of medicine) uses dry powder or crude extracts of plants to treat various disorders in humans including cancer. The observed effect is attributed not only to the single compound but also the other components present in the crude extract/s. The rationale for this type of treatment is that the toxicity of an active component may be counteracted by another component, which may not have the desired therapeutic property.

Aegle marmelos, commonly known as bael, is a spinous tree belonging to the family Rutaceae. It is widely found in India, Bangladesh, Burma and Sri Lanka. It is distributed mainly within the sub-Himalayan forests, in dry hilly regions ascending to 4000 ft. It is called “Shivadume”, the tree of Lord Shiva. Since ancient time, its leaves are offered to Lord Shiva and Parvathi. Aegle marmelos has an important place in indigenous systems of medicine. Its edible leaf, root, bark, seed and fruits are valued highly in Ayurvedic medicine in India. In fact as per Charaka (1500 BC) no drug has been longer or better known or appreciated by the inhabitants of India than the bael. The leaves of bael are astringent, laxative, febrifuge, and expectorant. They are useful in ophthalmia, deafness, inflammations, catarrh, diabetes, asthmatic complaints and weakness of heart. The unripe fruit is bitter, acid, sour, astringent, aids digestion and stomach irritation, and are useful in treating diarrhoea, dysentery, and stom-achalgia.

The roots of A. marmelos form an essential ingredient of dhamsmula (ten roots), a medicine commonly used by Ayurvedic practitioners. Fresh aqueous and alcoholic leaf extracts of Aegle have been reported to possess cardiotoxic effect like digitalis and decrease the requirement of circulatory stimulants. The aqueous decoction of the leaf has been shown to have a significant hypoglycemic effect. Aegle leaf extract has also been found to help in the regeneration of damaged pancreas (β-cells) in diabetic rats and is found to be as effective as insulin in restoring blood glucose and body weight to normal levels. Recently, the stem bark of bael has been reported to exhibit potent cytotoxic activities in human tumor cell lines. It has also been reported to possess cytotoxic effect on human breast cancer cell lines in vitro. However, the antineoplastic effect of the Aegle marmelos has not been investigated in vivo. Therefore, the present study was undertaken to obtain an insight into the antineoplastic effect of leaf extract of Aegle marmelos in Ehrlich ascites carcinoma bearing mice in vivo.

MATERIALS AND METHODS

Preparation of the Extract The Aegle marmelos (L.) CORREA, family Rutaceae was identified by Dr. Gopal Krishna Bhat (a well known taxonomist of this area) Department of Botany, Poorna Prajna College, Udupi, India. Mature leaves of Aegle marmelos were collected locally during January—February of the year. The leaves were thoroughly...
cleaned with at least three changes of water. The plant materials were shade dried, powdered, and extracted. Briefly, 100 g of the dry powders were separately extracted with 50% ethanol at 50 to 60 °C in a Soxhlet apparatus for 72 h. The cooled extract was concentrated to dryness in a lyophilizer. A 24% yield of the extract was obtained. The extract was stored in a freezer at −70 °C until use. Henceforth, the leaf extract of Aegle marmelos will be called as AME.

Anticancer Activity. Animal Care and Handling The animal care and handling was done according to the guidelines of World Health Organization, Geneva, Switzerland and the INSA (Indian National Science Academy, New Delhi, India). Ten to twelve weeks old female Swiss albino mice weighing 30 to 36 g were selected from an inbred colony maintained under the controlled conditions of temperature (23 ± 2 °C), humidity (50 ±5%) and light (14 and 10 h of light and dark, respectively). The animals had free access to the sterile food and water. Four animals were housed in a polypropylene cage containing sterile paddy husk (procured locally) as bedding throughout the experiment. The study was approved by the Animal Ethical Committee of Kasturba Medical College, Manipal.

Preparation of Drug and Mode of Administration The AME was dissolved in 100 μl of ethanol and diluted with the sterile physiological saline (SPS) as per the requirement. Tumor bearing mice was administered intraperitoneally with SPS or AME unless otherwise stated.

Acute Toxicity The acute toxicity of AME was determined according to Prieur et al. and Ghosh. Briefly, these animals were fasted by withdrawing food and water for 18 h. Fasted animals were divided into eight groups of 10 each. Each group of animals was injected intraperitoneally with 1000, 1500, 1750, 2000, 2125, 2250, 2375 and 2500 mg/kg body weight (b. wt.) of freshly prepared AME intraperitoneally. The animals were provided food and water immediately after the drug administration. Mortality of these animals was monitored up to 14 d post drug treatment.

Tumor Model Ehrlich ascites carcinoma (EAC) procured from the Cancer Research Institute (ACTREC), Mumbai, India, was maintained and propagated by serial intraperitoneal transplantation of EAC cells in an aseptic environment. 10^6 viable EAC cells were injected intraperitoneally into each animal in an aseptic condition and the day of tumor inoculation was considered as day 0. The animals were injected with various doses of AME as per the standard protocol recommended by the National Service Center of Cancer Chemotherapy, CCNSC, U.S.A.

Selection of Administration Schedule A separate experiment was carried out to evaluate the most effective administration schedule of AME. Twenty four hours after the EAC inoculation, the tumor bearing mice were divided into the following groups:

- SPS: The animals of this group received 0.3 to 0.35 ml of SPS once daily, for 6 consecutively days.
- AME: The animals of this group were injected with 400 mg/kg b. wt. of AME once daily, consecutively for 9 d.

Route of Administration Another experiment was carried out to evaluate the most effective route of administration of AME, where the tumor bearing mice were divided into the following groups:

- SPS: The animals of this group received 0.3 to 0.35 ml of SPS once daily, for 6 consecutively days.
- AME (i.p.): This group of animals received 400 mg/kg b. wt. of AME intraperitoneally once daily for 6 consecutive days.
- AME (p.o.): The animals of this group received 400 or 800 mg/kg b. wt. of AME orally once daily, consecutively for 6 d.

Selection of Optimum Dose A separate experiment was carried out to evaluate the best dose for the anticancer activity of AME when administered for 6 consecutive days, where the EAC bearing animals were divided into the following groups:

- SPS: The animals of this group received 0.3 to 0.35 ml of SPS.
- AME: The animals of this group were injected with 100, 200, 300, 400, 500 or 600 mg/kg b. wt. of AME once daily, consecutively for 6 d.

Alteration in Administration Schedule This experiment was carried out to evaluate the effect of change in the three administrations of 400 mg/kg b. wt. (1/10th of LD_{10} dose) of AME, during the whole study periods at different days as described below:

- SPS Control: The animals of this group received 0.3 to 0.36 ml of SPS, once daily for six consecutive days.
- AME 1–2–3: The animals of this group were administered with a single dose of 400 mg/kg b. wt. of AME once daily, consecutively for 3 d.
- AME 1–3–5: This group of animals was injected with a single dose of 400 mg/kg b. wt. of AME on day 1, 3 and 5, respectively.
- AME 5–7–9: The animals of this group received a single dose of 400 mg/kg b. wt. of AME on day 5, 7 and 9, respectively.

Stage Specific Evaluation The mice were inoculated with EAC cells and allowed to grow for 1, 3 and 6 d and for reasons of clarity these days have been designated as stage I, II, and III, respectively. The tumor bearing animals of various stages were divided into the following groups:

- SPS Control Group: The animals of this group received SPS at stage I, II or III.
- AME: The animals of this group were administered once daily with 400 mg/kg AME (the best dose) for 6 consecutive days at I, II or III stage of tumor development.

After the last administration of AME, the animals were monitored regularly for weight changes, signs of toxicity and mortality. The weights of animals were recorded every third day up to 30 d after tumor inoculation in all the groups. A 33% of drug related deaths or a weight loss of 5 g per mouse was considered as an index of toxicity. The animal survival was monitored daily up to 120 d, since the survival of animals up to 120 d is roughly equivalent to 5 years survival in man. The tumor response was assessed on the basis of median survival time and tumor free survival. The median survival time (MST) and the average survival time (AST) were
calculated from the animals dying within 120 d and those surviving 120 d were excluded from it. The increase in median life span (% IMLS) and increase in average life span (% IALS) were also calculated.

**Statistical Analysis** The statistical significance between the treatments was determined using “Z” test and the daily survival was determined by the Kaplan Meir’s equation.

**RESULTS**

**Acute Toxicity** The administration of 1000, 1500 or 1750 mg/kg b. wt. AME did not induce even a single death during the whole observation period. However, a further increase in the drug dose to 2000 mg/kg b. wt. resulted in a 10% reduction in mouse survival. An increase in AME dose to 2125 mg/kg b. wt. resulted in a 20% reduction in survival and 50% mice died when the drug dose was increased to 2250 mg/kg. A still further increase in the AME dose to 2375 mg/kg b. wt. resulted in 80% mortality and no animals survived when the AME dose was raised to 2500 mg/kg.

**Selection of Administration Schedule** The treatment of tumors with a single dose of 400 mg/kg of AME did not significantly alter the MST and AST (17 d) when compared with the SPS group, where it was found to be 16.2 and 17 d respectively (Fig. 1). However, there was an administration schedule dependent increase in the anticancer effect of AME and the best effect was observed when the drug was administered once daily, consecutively for six days as evident by an increase in both the MST and AST (Fig. 1), where they were found to be 29 and 27.4 d, respectively. However, a marginal increase in MST (17.5 d) and AST (18 d) was also observed when AME was administered for 3 d only. With a further increase in the number of administrations up to nine days, the toxic effects like debility, loss of body weight and death became manifest in the recipient animals (Fig. 2). Of all the administration schedules tested, the highest anticancer activity was observed for six administrations of 400 mg/kg and further studies were carried out using this schedule (Fig. 1).

**Route of Administration** The transplanted EAC tumors in the animals did not exhibit spontaneous regression throughout the experiments. The animals showed a constant weight gain due to tumor cell multiplication and growth (Fig. 3). The median survival time (MST) was found to be 17 in the SPS group, while the average survival time (AST) was 16 d (Fig. 3). Treatment of 24 h old tumors with 400 or 800 mg/kg of AME through the oral routes did not significantly alter the MST (16.5 and 17 d, respectively) and AST (16.7 and 17.1 d, respectively) when compared with the SPS group and all the animals succumbed to death by day 20 (Fig. 3). The AME administration through the intraperitoneal route retarded the increase in the tumor growth and the subsequent weight gain. It has increased the MST and AST by 12 and 11.2 d, respectively when compared with the SPS group. Consequently, this route of treatment caused an increase in the median life span (IMLS) and the average life span (IALS), which were found to be 70.58 and 69.1%, respectively (Fig. 3).
Selection of Optimum Dose  The treatment of 24 h old tumors with 100, 200, 300, 400, 500 or 600 mg/kg b. wt. of AME inhibited the weight increase in all the treatment group indicating arrest of tumor cell proliferation and growth (Fig. 4). However, administration of 500 and 600 mg/kg AME was also accompanied by some toxic side effects like ruffling of hair, sluggishness and lacremation in the recipients and none of the animals treated with 600 mg/kg group survived beyond day 9 post-tumor inoculation (Fig. 6). The administration of 100 or 200 mg/kg AME did not significantly alter the MST (19 and 20 d, respectively) and AST (18 and 19.4 d, respectively) when compared with the SPS group. The greatest effect was observed for the animals treated with 300 or 400 mg/kg AME, where the MST increased up to 24 and 28.5 d, respectively when compared with the SPS treated group. The AST was also elevated up to 22.5 and 27.7 for 300 and 400 mg/kg AME, respectively. Of all the AME doses tested, the highest anticancer activity was observed for 400 mg/kg, where prolongation of life of animals was observed till day 43 post tumor inoculation (Fig. 5). This dose also did not induce any toxic effects in the form of debility, loss of body weight and death and further studies were carried out using this dose of ASE. The IMLS and IALS was observed to be 67.6% and 70.98% respectively for 400 mg AME (Fig. 6). Apart from 6 d consecutive treatment with AME, the EAC mice were injected with 100, 200 and 300 mg/kg AME for consecutive 24, 12 and 8 d, respectively. However none of these regimens were effective in inhibiting the tumor growth as the survival of tumor bearing mice was not prolonged when compared to 400 mg/kg AME given for 6 consecutive days.

Alteration in Administration Schedule  The animals transplanted with EAC cells did not show spontaneous regression of tumors, which was evident by a constant gain in weight and increase in the tumor volume due to tumor cell multiplication and growth. The MST was found to be 17 d for the SPS group, while the AST was 16.2 d. The administration of 400 mg/kg b. wt. of AME on day 1–2–3 or 1–3–5 or 1–5–9 inhibited the tumor growth, which is evident by a delay in the weight gain due to the tumor cell multiplication and growth in the recipients. Triple administration schedule failed to prolong the life span of tumor bearing animals. The MST was 17.5 (AME 1–2–3), 17 (AME 1–3–5) and 16 (AME 1–5–9), while AST was 18 (AME 1–2–3), 17.4 (AME 1–3–5) and 16 (AME 1–5–9) d.

Stage Specific Evaluation  The stage specific evaluation of the anticancer activity of AME was carried out in tumor bearing animals on 1, 3 or 6 d, (stage I, II and III, respectively) by administering 400 mg/kg AME for 6 d, consecutively at stage I, II or III. AME was effective in reducing the weight gain in the animals due to tumor development, especially during the early stages only (Fig. 7). Treatment of mice with 400 mg/kg AME at various stages of tumor development resulted in an increase only in the early stages (Fig. 8). The MST increased up to 29, 20 and 17.5 d and AST to 27.8, 20.3 and 17 at stage I, II or III, respectively (Fig. 9). The best effect was observed when AME was administered on stage I only. The IMLS and IALS declined depending on the treatment stage and the lowest values were observed for stage III.
The IMLS of 70.5, 17.6 and 3% while the IALS of 70.3, 25.3 and 5% was found for stage I, II and III, respectively.

DISCUSSION

The two fundamental cancer treatments, chemotherapy and radiotherapy, long been known to have a magnitude of short and long term adverse effects. The principle objectives of combining chemotherapy and radiotherapy are to increase local tumor control, decrease distal metastasis and improve survival. Many drugs are being used as chemotherapeutic agents against various forms of cancer. However, most of the agents invariably have cell toxicity and can induce genotoxic, carcinogenic and teratogenic effects in non-tumor cells that can give rise to secondary tumors. In recent years many natural compounds derived from plants and/or crude plants extracts have been proved to have protective effect against toxic effects of many chemicals and to combat a variety of ailments.18–20) Plants have a long history of use in the treatment of cancer.21,22) The need to find a safe and highly effective cure for neoplastic diseases remains a major challenge for modern medicine. Flavones, flavanols, isoflavones, catachins and tannins present in many plants have also been shown to possess anticarcinogenic and antimutagenic potential. Furthermore, some of the herbal medicines and their constituents have been reported to inhibit the proliferation of cancer cells directly and also have been found to be clinically useful.23) The need for newer paradigms for cancer treatment cannot be underestimated as reduction in mortality rates due to cancer is still an elusive goal. Therefore, an attempt has been made to evaluate the anticancer activity of the leaf extract of *Aegle marmelos*, which is commonly used in Ayurvedic system of medicine for various purposes.

The treatment of animals with AME up to a dose of 1750 mg/kg did not induce toxicity and no drug-induced mortality was observed. The LD₁₀ and LD₅₀ for the drug induced acute mortality was found to be 2000 and 2250 mg/kg b. wt. respectively. The AME has been reported to be non-toxic up to 4 g/kg orally.24) The triple administration of 400 mg/kg AME (1/5th of LD₁₀) at different days of the tumor progression gives a clear idea about the stage at which the drug is effective in restricting the tumor cell proliferation and therefore, three different schedules of triple administrations were selected. The results indicate that the antineoplastic effect was pronounced only in the early stages of the tumor development than at the late stages and the best effect was observed for 1–2–3, followed by 1–3–5 and 1–5–9 days, respectively. The reason for this observed trend may be that the drug may be more effective in arresting tumor growth at the early stages than the later stages of the tumor growth. As far as the authors are aware, this type of regime has not been performed earlier and hence it may not be possible to discuss these findings in lieu of the others observations.

The spatial effect of drug administration was evaluated using various drug schedules of administrations. The single administration of AME on day one or for three days was in-
effective, as these durations did not enhance the survival of mice when compared with the untreated control group. However, when the drug was administered once daily for six days, there was a dramatic increase in the survival of tumor-bearing mice and the highest number of survivors were observed in the group of animals that received single injection of AME, consecutively for six days. Reports regarding the anti-neoplastic action of AME in vivo are unavailable. However it has been reported to inhibit the in vitro proliferation of human tumor cell lines like leukemia K562, T-lymphoid Jurkat, B-lymphoid Raji, erythroleukemic HEL, melanoma Colo38, breast cancer MCF7 and MDA-MB-231. Skimmianine, an alkaloid present in Aegle marmelos has been found to show cytotoxic activity against the A 2780 human ovarian cancer cell lines. The other medicinal plants like Ervatamia heynicana, Rubia cordifolia, Hygrophiila spinosam, Tylophora indica, Podophyllum hexandru and Hygrophiila spinosa have been reported to possess antineoplastic activity. The administration of AME for nine days induced toxic effects in the form of debility, loss of body weight and death and did not prolong survival. Therefore 6 d regimen was considered best for the antineoplastic activity of AME and further experiments were carried out with this regimen.

Treatment of animals with AME up to a dose of 400 mg/kg, daily once for six consecutive days did not induce toxicity and no drug-induced mortality was observed. The highest drug doses screened in this study 500 and 600 showed toxic manifestations like diarrhea, body weight loss and mortality. However no such symptoms were associated up to 400 mg/kg. Therefore, 400 mg/kg AME dose, which produced highest anticancer effect was considered as an optimum dose. The reason for this may be that after a particular concentration, the drug instead of being effective may become toxic to the vital organs (like heart, GI, kidney, liver, lungs and the brain). The finding that the animals are able to tolerate cumulative dose of 2400 mg (400 mg/kg, for 6 d) without serious side effects is significant from the clinical point.

The clinical efficacy of an anticancer agent lies in its ability to inhibit the proliferation of tumors not only in early stages but also in the late stages of its development. The results from the stages specific evaluation show that AME inhibited the increase in the body weight gain in animals due to tumor development during the early stages effectively, which may be due to an efficient tumor cell kill by AME. The studies of the anticancer activity of plants at different stages of tumor development are scanty, however, withaferin A from Withania somnifera and plumbagin, from Plumbago rosea have been reported to inhibit EAC growth when administered in mid stages.

In conventional chemotherapy, it is important to know a drug’s efficacy through oral routes as this is most convenient and does not require medical intervention in the cancer treatment; therefore, the drug was administered orally at two doses (400 or 800 mg/kg) apart from the intraperitoneal route. Our results show that the best antineoplastic action of AME was obtained when the drug was administered intraperitoneally, while the oral route was not as effective as the intraperitoneal route. The ineffectiveness of oral route at this dose may be due to the less availability of the drug to the tumor cells as the drug passes through the digestive system and only a fraction of the administered drug may have reached the target. However, it is logical to assume that when the drug is administered through the intraperitoneal route, it comes in direct contact with the tumor cells and therefore, it is highly effective in causing arrest in growth and multiplication of the EAC cells. Similar observations have been reported earlier for other plant extracts like Withania somnifera and Plumbago rosea.

From our study it is clear that AME inhibited the growth of EAC in the mice. The optimum non-toxic dose of 400 mg/kg was 1/5.6th of the LD50 (2250 mg/kg) dose and the non-toxic nature of this drug may lie in its composite status. The exact mechanism of action of AME is not known. It may be due to operation of multiple events. AME administration may have inflicted irreparable damage to the cell DNA, which may have further inhibited the division of tumor cells leading to cell killing. AME contains imperatorin that has been reported to show antiproliferative effect on several cancer cell lines. The imperatorin present in AME has been reported to induce cytochrome c-dependent apoptosis in human promyelocytic leukemia, HL-60 cells. Therefore induction of apoptosis by AME may have killed the tumor cells.

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