Tests for Antinociceptive Effect of Methanolic Extract of *Aegle marmelos* Bark

This thesis paper is submitted to the Department of Pharmacy, East West University for the Degree of Bachelor of Pharmacy

Submitted by

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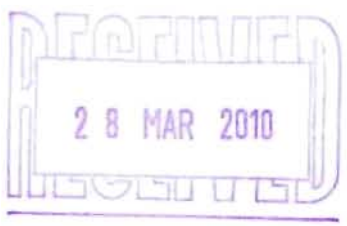

Certificate

This is to certify that, the thesis, "Tests for Antinociceptive Effect of Methanolic Extract of *Aegle marmelos* Bark" submitted to the Department of Pharmacy, East West University, Mohakhali, Dhaka, in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (B. Pharm) was carried out by Tahmina Khanam (ID# 2005-2-70-083) under our guidance and supervision and no part of the thesis has been submitted for any other degree. We further certify that all the sources of information and laboratory facilities of in this connection is duly acknowledged.

27/12/09

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Dedicated
To my

Parents

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Running head: ANTINOCICEPTIVE EFFECT OF *A. marmelos*

Tests for Antinociceptive Effect of Methanolic Extract of *Aegle marmelos* Bark

Tahmina khanam (ID-2005-2-70-083)
East West University

In partial fulfillment of the requirements

for

Degree Bachelor of Pharmacy

Abu Taiab Md. Jamaluddin

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Abstract

An experiment was conducted to determine the antinociceptive effect of methanolic extract of *Aegle marmelos* bark in swiss albino mice. The nociceptive effect was induced in test animals by administering 0.6% Acetic acid solution intraperitoneally. Control group received normal saline (10ml/kg i.p) and diclofenac (10mg/kg i.p) was used as a reference drug. The result has shown that oral administration of *Aegle marmelos* bark extract (250 mg/kg and 500 mg/kg) produced antinociception against acetic acid-induced writhing. The percent inhibition of writhing observed by the bark extract administered at a dose of 250 mg/kg was 73.89% (p=0.0); and that for the dose of 500 mg/kg was 97.34%(p=0.000008); the latter result was even higher than the data showed by the positive control diclofenac (88.49%). Therefore the present results suggest that methanolic extract of *Aegle marmelos* bark may play a role in reducing pain or can exhibit antinociceptive action in mice.

Key words: methanolic extract, *Aegle marmelos*, positive control, negative control, Albino mice, writhing, antinociceptive.

Introduction

Aegle marmelos is belongs to the family Rutaceae, commonly called as Bael (English), and is found throughout India and Bangladesh . Bael is a medium sized decidous tree bearing strong axillary thorns. Leaves with 3 or 5 leaflets. Bael (*Aegle marmelos*) is a fruit-bearing tree indigenous to dry forests on hills and plains of central and southern India, southern Nepal, Sri Lanka, Myanmar, Pakistan, Bangladesh, Nepal, Vietnam, Laos, Cambodia and Thailand.

It is cultivated throughout India, as well as in Sri Lanka, northern Malay Peninsula, Java and in the Philippines. It is also popularly known as Bilva, Bilwa, Bel, Kuvalam, Koovalam, Madtoum, or Beli fruit, Bengal quince, stone apple, and wood apple. The tree, which is the only species in the genus *Aegle*, grows up to 18 meters tall and bears thorns and fragrant flowers. It has a woody-skinned, smooth fruit 5-15 cm in diameter. The skin of some forms of the fruit is so hard it must be cracked open with a hammer. It has numerous seeds, which are densely covered with fibrous hairs and are embedded in a thick, gluey, aromatic pulp.

Fig 01: Fruit at Narendrapur near Kolkata.

Fig 02: Aegle marmelos trunk

Table 01: Scientific classification of *Aegle marmelos*

Scientific classification	
Kingdom:	Plantae
Order:	Sapindales
Family:	Rutaceae
Subfamily:	Aurantioideae
Genus:	*Aegle*
Species:	*A. marmelos*

The fruit is eaten fresh or dried. If fresh, the juice is strained and sweetened to make a drink similar to lemonade, and is also used in making sharbat, a refreshing drink where the pulp is mixed with lime juice. If the fruit is to be dried, it is usually sliced first and left to dry by the heat of the sun. The hard leathery slices are then placed in a pan with several litres of water which is then boiled and simmered. As for other parts of the plant, the leaves and small shoots are eaten as salad greens.

The fruit is also used in religious rituals and as an ayurvedic remedy for such ailments as diarrhea, dysentery, intestinal parasites, dryness of the eyes, and the common cold. It is a very powerful antidote for chronic constipation.

In the traditional culture of Nepal, the Bael tree is part of an important fertility ritual for girls known as the *Bel baha*. This tree is a larval foodplant for the following two Indian Swallowtail butterflies:

The **Lime** Butterfly: *Papilio demoleus.*

The Common Morm. (Bakhru, 1997).

Chemical Constituents and Components

Main chemical components are marmelosin, alloimperatorin, marmelide, tannic acid, marmin, umbelliferone, isoimperatorin, isopimpinellin, skimmin, marmesin, marmesinin, fatty acids, beta-sitosterol

Action

Mucilage

It increases the glucose level and glycosylated hemoglobin in diabetic patients.

It decreases plasma insulin and liver glycogen in diabetic patients.

It decreases the lipid peroxidation.

It stimulates macrophage functioning.

It causes significant elevation in the GSH (glutathione) concentration in liver, kidney, stomach, and intestine.

Tannins

It shows potent anti-viral activity.

It causes significant decrease in lipid peroxidation, conjugated diene and hydroperoxide levels in serum.

It significantly reduces the blood sugar level.

It reduces the significant oxidative stress.

Curing Diseases

Sweet drink prepared from the pulp of fruit produce soothing and cooling effect.

The unripe and half-ripe fruits improve appetite and digestion.

The ripe fruit is a good and simple cure for dysentery and dyspepsia.

The roots and bark of the tree are used in the treatment of fever and malaria.

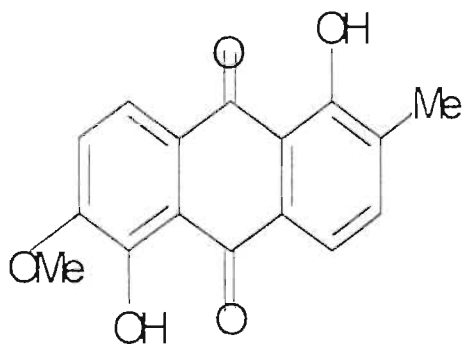
The roots are used to cure pain and palpitation of the heart.

Various parts of *Aegle marmelos*, Correa, Family Rutaceae, commonly known as Bael, are used in Ayurveda, as well as in Unani medicine for the treatment of many diseases. Unripe fruit of the plant is prescribed in chronic diarrhoea and dysentery. Decoction of the root is useful in intermittent fever, hypochondriasis, melancholia and palpitation of the heart. The plant possess antifungal activity, antibacterial activity, radioprotective effect and anticancer activity. The Bael leaves can be used as potential hypoglycemic agent (Kamalakkanan, 2003).

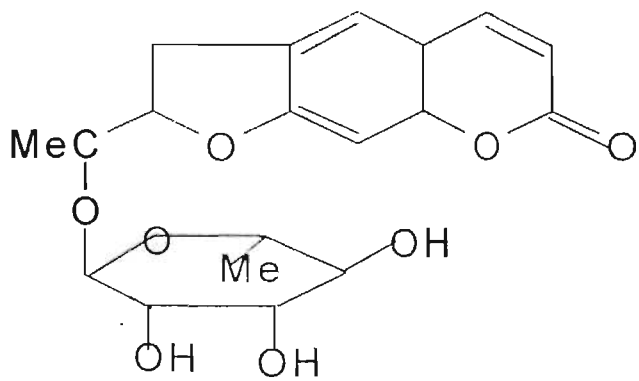
Scientists studied the isolation and constitution of marmin, a new coumarin from *Aegle marmelos* umbelliferone, skimmianine and a sitosterol, were isolated from the immature bark of *Aegle marmelos*. The constitution of was established as 7- (3, 7 dihydroxy-3, 7-dimethyloctyloxy) coumarin (Chatterjee & Mitra, 1949).

The *A. marmelos* fruit pulp has been shown to posses antiprotozoal activity in chronic dysentery condition accompanied by loose stool alternately with occasional constipation. (Singh & Chaturvadi, 1981). The ripe fruit used in different formulation for treatment of chronic diarrhea.

On the year 1991, isolation of new pigment from stem bark of the *Aegle marmelos* was done..The isolation and structure elucidation of new compound is marmesin – 1"- α -L - rhamnopyranoside and 1,5 -dihydroxy - 6 - methoxy -2 -methyl anthraquinone, which occur together with lupeol and β-sitosterol in the stem bark of *Aegle marmelos* were describes. V.K. Gupta *et al* studied the sample coumarin compound R- (+) – marmin from the trunk bark of the *Aegle marmelos* by methanol extract. Then extracted compound concentrated and chromatographed over the silica gel, and the chemical structure were assigned on the basis of the H^+ NMR and mass spectra. (Nema & Srivastava, 1991)



1,5-dihydroxy-6-methoxy-2-methylanthraquinone



Marmesin-1[1]-α - L-rhamnopyranoside

Fig 03: 1,5-dihydroxy-6-methoxy-2-methylanthraquinone and Marmesin-1[1]-α - L-rhamnopyranoside

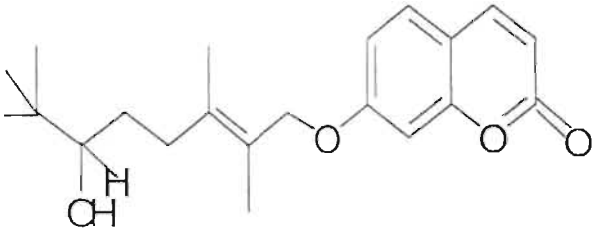


Fig 04: R- (+) marmin

Isolation of two new 7-geranyloxycoumarins from the bark of the *Aegle marmelos* was also a major breakthrough. Two new 7-gerayloxycoumarins and aeglin, were isolated from the bark of *Aegle marmelos*, and those structures were assigned on the basis of the NMR data .The absolute configuration was confirmed by chemical synthesis. (Ohashi, 1994).

Ohashi also isolated four isomeric lignan-glucosides from the bark of *Aegle marmelos*. Two new lignan – glucoside, (-) – lyoniresinol 2α-O-β-D glucopyranoside and (-) 4 - epi-lyoniresinol, 3α -O -β -D-glucopyranoside, have been isolated together with two known lignan - glucosides, (+)- lyoniresinol. 3α-O-β-D-glucopyranoside and (-)-lyoniresinol 3α-O-β-D-D-glucopyranoside. (Ohashi, 1995).

Evaluation of the anti fungal activity of essential oil isolated from leaves of the *Aegle marmelos* was done using spore germination assay. (Rana & Jain, 1997). The oil established variable efficacy against different fungal isolation and 100% spore germination off all the fungi tested and observed 500 PPM. However the most resistant fungus, F*usarium udum* was inhibited 80% at 400 PPM.

Scientists named Riyanto and Mustafa isolated few compound from *A. marmelos* bark from petroleum ether extract. The crude extract was subjected to column chromatography. The isolated compounds were identified on the basis various spectral data (UV, IR, Mass and NMR). Two triterpenes, lupenone and lupeol were obtained in the form of white crystal. (Riyanto, 2000).

Aritajat and co-workers confirmed dominant lethal test in rats treated with some plant extracts. . (Aritajat & Kaweewat, 2000). They investigated the toxic effect of aqueous extracts of *Aegle marmelos* (AM) and other plant extracts on rats by dominant lethal test. The data of 8-week treatment suggested that none of the extracts adversely affected male body and testicular weights as well as cauda epididymal sperm counts. No notable changes in sperm morphology and motility were observed. There were no abnormal changes in the number of implantation sites, number of viable fetuses and number of dead fetuses in females mated with plant extract-treated males relative to controls. Based on these results, it could be concluded that all the investigated plant extracts have no toxic effect on male rat reproduction and progeny outcome.

Antidiarrhoeal activity of *Aegle marmelos* unripe fruit and other plants in castor-oil induced diarrhoea was observed by Shoba and Thomas (Shoba, 2001). A study was

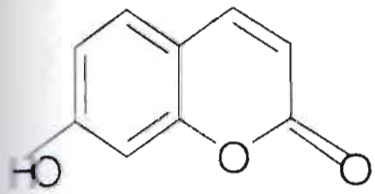
undertaken to evaluate the effect of aqueous and methanolic plant extracts of *Acorus calamus* rhizome, *Pongamia glabra* leaves, *Aegle marmelos* unripe fruit and *Strychnos nux-vomica* root bark for their antidiarrhoeal potential against castor-oil induced diarrhoea in mice. The methanolic plant extracts were more effective than aqueous plant extracts against castor-oil induced diarrhoea. The methanolic plant extracts significantly reduced induction time of diarrhoea and total weight of the faeces. The result obtained establishes the efficacy of these plant extracts as antidiarrhoeal agents.

Two biochemists named Kamalakkannan and Stanely Mainzen Prince used the notion that *Aegle marmelos* Corr. (Rutaceae) is widely used in Indian Ayurvedic medicine for the treatment of diabetes mellitus and conducted a pharmacology based research to prove it. The hypoglycaemic effect of the water extract of the fruits of *Aegle marmelos* was examined in streptozotocin-induced diabetic Wistar rats. Oral administration of the water extract (125 and 250 mg kg^{-1}) twice a day for 4 weeks resulted in significant reductions in blood glucose, plasma thiobarbituric acid reactive substances, hydroperoxides, ceruloplasmin and α-tocopherol and a significant elevation in plasma reduced glutathione and Vitamin C in diabetic rats. The effect of the extract at a dose of 250 mg kg^{-1} was more effective than glibenclamide in restoring the values of these parameters. The results of this study clearly shows the hypoglycaemic activity of the fruit extract. (Kamalakkannan, 2003).

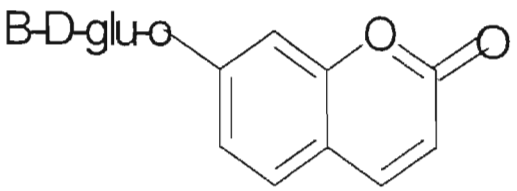
Investigation on the gastroprotective and antidiarrhoeal properties of *Aegle marmelos* unripe fruit extract was studied by Dhuley JN. The Study was designed to verify the gastroprotective and antidiarrhoeal effects of unripe fruit extract of *Aegle marmelos*

Corr. The gastroprotective function of this extract was evaluated in rats against gastric mucosal damage induced by hypothermic restraint stress, absolute ethanol, and indomethacin, whereas the antidiarrhoeal activity was investigated by studying the influence on gastrointestinal transit as measured by a charcoal marker and on castor oil-induced accumulation of intestinal fluid in mice and also on contractile responses evoked by acetylcholine, histamine, serotonin, and barium chloride in isolated guinea-pig ileum, the results demonstrated that pretreatment of animals with unripe fruit extract (50 and 100 mg/kg, i.p.) produces a significant inhibition of gastric lesion induced by ethanol but not those induced by restraint stress or indomethacin and suggest a probable involvement of a prostaglandin-independent mechanism of gastroprotection. At similar doses, both the intestinal transit as well as the accumulation of intestinal fluids induced by castor oil in mice was significantly inhibited by raw fruit extract. Furthermore, the extract antagonized the contractile responses evoked by different agonists on guinea-pig ileum *in vitro* and its inhibitory potential for the drugs are in the order of acetylcholine > histamine > serotonin > barium chloride. Taken together, these results point out a possible antidiarrhoeal effect of unripe fruit extract of *A. marmelos* Corr., since inhibition of intestinal motility and secretion can control clinical diarrhoea. (Dhuley, 2003).

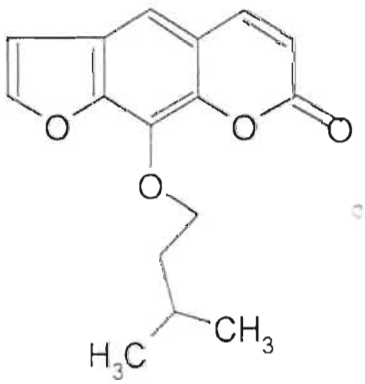
Samarasekera isolated various coumarin present in the various part of the *Aegle marmelos*. These are Umbeliferone, Skimmin, Impertonin. (Samarasekera, 2004). The structures of these coumarins are given below.



Umbeliferone



Skimmin



Impertonin

Fig 05: Umbeliferone, Skimmin, Impertonin

Hypoglycemic and antioxidant activity of *Aegle marmelos* in alloxan induced diabetic rats was studied by Upadhya and co-workers. Male albino rats were randomly divided into three groups: Group I: Control; Group II: Diabetic rats; and Group III: Diabetic rats administered AML. Glucose, urea and glutathione-S-transferase (GST) in plasma, glutathione (GSH) and malondi-aldehyde (MDA) levels in erythrocytes were estimated in all the groups at the end of 4 weeks. There was a decrease in blood glucose at the end of four weeks in-group III animals compared with group II, however it did not reach the control levels. There was an increase in erythrocyte GSH and a decrease in MDA in group III as compared to group II. The plasma GST levels were raised in diabetic rats

when compared to controls. In the group III animals, there was a decrease in GST as compared to group II. Owing to hypoglycemic and antioxidant properties, AML may be useful in the long-term management of diabetes. (Upadhya, 2004).

A new 7-geranyloxycoumarin [7-(2,6-dihydroxy-7-methoxy-7-methyl-3-octaenyloxy) coumarin] named marmenol has been isolated from the leaves of methanolic extract of *Aegle marmelos*. In addition to marmenol, several known compounds have also been obtained for the first time from the same source. They include: praealtin D, *trans*-cinnamic acid, valencic acid, 4-methoxy benzoic acid, betulinic acid, N-*p*-*cis*- and *trans*-coumaroyltyramine, montanine, and rutaretin. (Muhammad, 2004).

On the same month, A new insecticidal protolimonoid from *Aegle Marmelos* was discovered by Radhika Samarasekera, Bhupinder and coworkers. Their research result depicted that the Bioassay-directed fractionation of the ethyl acetate extracts of the stem bark of *Aegle marmelos* Correa afforded a new compound, named skimmiarepin C, along with skimmiarepin A. The latter is a known compound but isolation from *A. marmelos* is new. The new compound is a senecioate ester analogue of the latter. Skimmiarepins A and C exhibit moderate insecticidal activity against *Phaedon cochleariae* and *Musca domestica* in comparison with natural pyrethrum extract. The two epimeric acetates of skimmiarepin C are both less active. (Radhika, 2004).

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. 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 LD_{50} dose, which increased the MST and AST up to 29 and 27 d, respectively. (Jagetia, 2004).

The radioprotective effect of bael (*Aegle marmelos*, AME) extract was studied in Swiss albino mice against radiation-induced changes in the peripheral blood, spleen colony forming units, and intestinal mucosa. The results of study related that AME pretreatment significantly decreased lipid peroxidation accompanied by a significant elevation in the GSH concentration in the mouse intestine. The data clearly indicate that the AME significantly reduced the deleterious effect of radiation in the intestine and bone marrow of mouse and could be a useful agent in reducing the side effects of therapeutic radiation. (Jagetia, 2005).

Leticia and Costa evaluated the anticancer potential used in Bangladeshi folk medicine. The extracts of *Aegle marmelos* were tested for cytotoxicity using brine shrimp lethality assay; sea urchin eggs assay, and MTT assay using tumor cell lines. The extract of *Aegle marmelos* exhibited toxicity on all used assays. . (Latica, 2005).

Veera Sumanth and Mustafa worked with ethanolic extract of unripe fruits of *Aegle marmelos* and tested for antistress, adoptogenic activity in mice using swim endurance test and cold restrain stress. An Adaptogen increases the power of resistance against physical, chemical or biological noxious agents; it has a normalizing influence on body. The mode of action of adoptogens is basically associated with the stress system Adoptogens increase the capacity of stress system to respond to external signals at the higher level of the equilibrium of activating and deactivating mediators of stress response. The plant Adoptogen is defined as "Smooth pro-stressors which reduce reactivity of host defense systems and decrease damaging effects of various stressors due to increased basal level of mediators involved in the stress response". A number of plants possess adoptogenic activity due to diverse classes of chemical compounds. The extract exhibited significant antistress, adoptogenic activity by improving the swim duration and reducing the elevated WBC, blood glucose and plasma cartisone. Acute toxicity studies revealed that LD_{50} is more than a dose of 3 g/kg body weight.(Sumanth, 2005).

Influence of selected Indian immunostimulant herbs including *Aegle marmelos* against white spot syndrome virus (WSSV) infection in black tiger shrimp was studied by Citarasu T The methanolic extracts of *Aegle marmelos* and other plants revealed that the application of herbal immunostimulants had been effective against shrimp viral pathogenesis and they can be recommended for shrimp culture. (Citarasu, 2006).

Das U K used aqueous extract of the *A. marmelos* as per the dose 50mg/100gm body wt and resulted with a significant determination in the key testicular steriogenic enzyme

along with low level of plasma testosterone and relative wt of the sex organs in respect to counter without any significant alternative in general body growth. (Das, 2006).

Miyazaki et all used total alcoholic, total aqueous, whole aqueous and methanolic extracts isolated from the leaves of *Aegle marmelos* and studied their toxic effects. Acute, sub-acute and LD_{50} values were determined in experimental rats. The dead animals were obtained from primary screening studies, LD_{50} value determination experiments and acute studies subjected to postmortem studies. The external appearance of the dead animals, the appearance of the viscera, heart, lungs, stomach, intestine, liver, kidney, spleen and brain were carefully noted and any apparent and significant features or differences from the norm were recorded. Collectively, these data demonstrate that the extracts of the leaves of *A. marmelos* have a high margin of drug safety. (Miyazaki, 2007).

Sudharameshwari and Radhika worked on the antimicrobial activity of *Aegle marmelos*, it was extracted by soxhlet apparatus using petroleum ether, ethanol, chloroform and aqueous as solvent. Among those extracts, the petroleum ether was considered as effective one. The extracts were subjected to preliminary phytochemical screening and the plant with four extracts were tested against three Gram positive bacteria (*B.cereus*, *B subtilis*, *S. aureus*) and three Gram negative bacteria (*E.coli, P.vulgaris,* and *P aeruginosa*) by disc diffusion method. The zone of inhibition of the extracts was compared with the standard antibiotics Streptomycin and Spectinomycin.. The phytochemical screening was done by subjecting the extracts to Preliminary phytochemical Screening methodology which were adapted from Kemp (1986) and

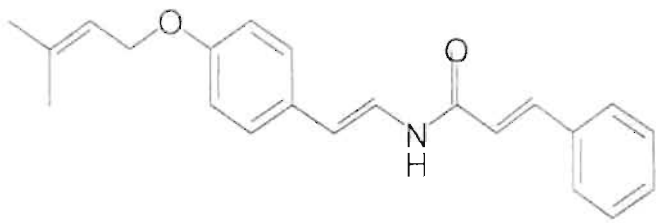
Sofowara (1982) method. Chloroform extract of *Aegle marmelos* shows the presence of alkaloids. The presence of flavanoides was determined using one ml of extract was added with a few drops of neutral ferric chloride solution. The extract was also tested for anthrocyanin by adding a drop of concentrated Sulphuric acid to one of extract. Flavanoids is present only in the petroleum ether extract of *Aegle marmelos.* Petroleum ether extract of *Aegle marmelos*, ethanol extract of *Aegle marmelos* indicate the presence of anthocyanin. The presence of coumarins was determined as well as phenols, steroids, phytosterols, xanthoproteins and Saponins in the chloroform extract of *Aegle marmelos*, petroleum, Chloroform and ethanol extract of *Aegle marmelos.* (Sudharameshwari, 2007).

For observing the antimicrobial activity with four extracts were tested against six pathogenic bacterial strains, three Gram positive bacteria (*B.cereus, B. subtilis, S aureus)* and three Gram negative bacteria(*E. coli. P .vulgaris, S.aureus* and *P aeruginosa)* by disc diffusion method. The antibacterial activity was recorded by measuring the width of the clear inhibition zone around each disc. The maximum antibacterial efficiency was found to be present in petroleum ether extract of *Aegle marmelos*. Petroleum ether extract of *Aegle marmelos* has showed no activity against *B. cereus* compare to other plants. No activity was found to be against *P.aeruginosa* and *S.aureus* in chloroform extract of *Aegle marmelos*. Similar result was obtained from the antibacterial activity of Aegle *marmelos*. Among the four solvents used in the study, petroleum ether was considered as the effective one. Because the petroleum ether extract exhibited maximum zone of inhibition against all pathogens compare to the other solvents. (Sudharameshwari, 2007).

isolation of 24-epibrassinolide from leaves of *Aegle marmelos* and evaluation of its antigenotoxicity employing *Allium cepa* chromosomal aberration is assayed by Nishi Sondhi, Renu Bhardwaj and their coworkers. They found that brassinosteroids are of universal occurrence in plants. They have been reported to affect plant growth and development through a spectrum of physiological responses. Recently they are reported to confer resistance in plants against a number of biotic and abiotic stresses. In the present study, a brassinosteroid was isolated from *Aegle marmelos* Correa. (Rutaceae) which was characterized to be 24-epibrassinolide (EBL) using various spectroscopic techniques (TLC and ESI-MS analysis). It was evaluated for the antigenotoxicity against maleic hydrazide (MH) induced genotoxicity in *Allium cepa* chromosomal aberration assay. It was shown that the percentage of chromosomal aberrations induced by maleic hydrazide (0.01%) declined significantly with 24-epibrassinolide treatment. EBL (10^{-7} M) proved to be the most effective concentration with 91.8% inhibition. This is the first report on the isolation of 24-epibrassinolide from *Aegle marmelos* and its antigenotoxic effects against MH employing *Allium cepa* chromosomal aberration assay. (Nishi, 2007).

On 2008, Dhalwal and his coworkers examined the Antioxidant Profile and HPTLC-Densitometric Analysis of Umbelliferone and Psoralen in Aegle marmelos. They reported a successful antioxidant activity of umbelliferone and psoralen in a methanol extract of A. marmelos. fruit pulp. A simple high performance thin layer chromatography (HPTLC) method has been developed for the simultaneous quantification of umbelliferone and psoralen. The results obtained from the current study suggest that A. marmelos. fruit is a potential source of natural antioxidants. (Dhalwal, 2008).

A new series of α-glucosidase inhibitors from the leaves of *Aegle marmelos* had discovered by Preecha Phuwapraisirisan of Natural Products Research Unit in Thailand and his coworkers. They reported presence of a series of phenylethyl cinnamides, which included new compounds named anhydromarmeline, aegelinosides A and B, were isolated from *Aegle marmelos* leaves as α-glucosidase inhibitors. The structures of new compounds were characterized by spectroscopic data and chemical degradation. Of compounds isolated, anhydroaegeline revealed the most potent inhibitory effect against α-glucosidase with IC_{50} value of 35.8 μM. The present result also supports ethnopharmacological use of *A. marmelos* as a remedy for diabetes mellitus. (Preecha, 2008).



Anhydromarmeline

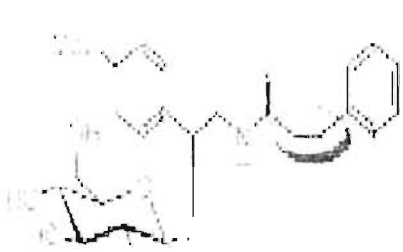


Fig 06: Anhydromarmeline; Aegelinosides A; Aegelinosides B. (Preecha, 2008).

On December, 2008, a novel and highly labile oxazoline from *Aegle marmelos* have been reported by Shaheen Faizi , Fatima Farooqi and their coworkers. They reported a rare alkaloid, shahidine(1), having an unstable oxazoline core has been isolated as a major constituent from the fresh leaves of *Aegle marmelos*. It is moisture-sensitive, and found to be the parent compound of aegeline and other amides, however, it is stable in dimethyl sulfoxide. Its structure was established by spectroscopic analysis. Biogenetically, oxazolines may be considered as the precursor of hydroxy amides and oxazoles found in plants. Shahidine showed activity against a few Gram-positive bacteria. (Shaheen, 2008).

Shahidine(1)

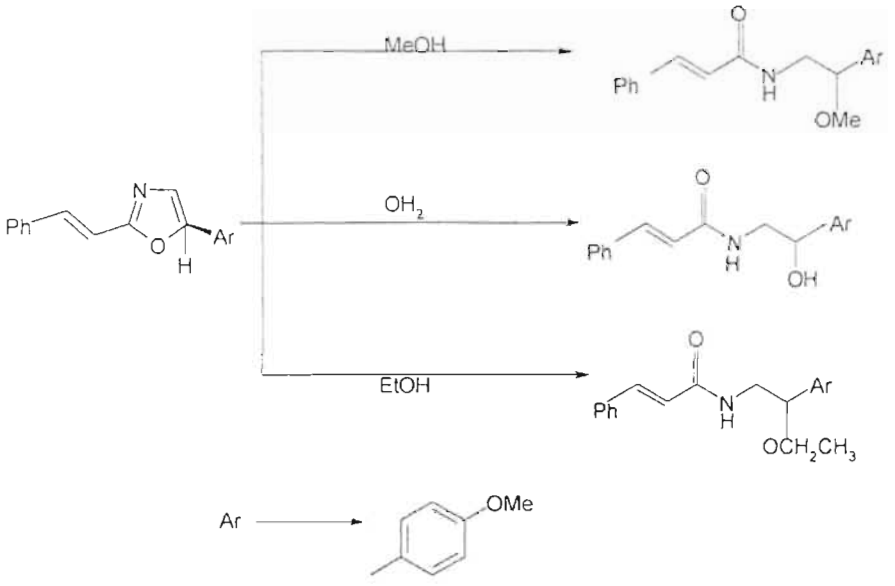


Fig 07: Graphical Abstract of Shahidine and its derivatives

Another research report conducted by *Vijaya and Ramanathan proved the* Lipid lowering activity of ethanolic extract of leaves of Aegle marmelos (Linn.) in hyperlipidaemic models of Wistar albino rats.Lipid lowering effect of 50% ethanolic

extract of the leaves of A. marmelos (Linn.) was evaluated in triton and diet induced hyperlipidaemic models of Wistar albino rats. The extract at 125 and 250 mg/kg dose levels inhibited the elevation in serum cholesterol and triglycerides levels on Triton WR 1339 administration in rats. The extract at the same dose levels significantly attenuated the elevated serum total cholesterol and triglycerides with an increase in the high-density lipoprotein cholesterol in high-fat diet- induced hyperlipidaemic rats. The standard drugs atorvastatin in the former and gemfibrozil in the latter studies showed slightly better effects. (Vijaya, 2009).

Summary of The Research Findings with *Aegle marmelos*

Table 02 : Phytochemical research findings

Time Chronicles	Name of the researchers	Part of the plant	Findings
1949	Chatterjee, A, and Mitra, S S.	Immature bark of *Aegle marmelos*.	Isolation and constitution of marmin as 7- (3. 7 dihydroxy-3, 7-dimethyloctyloxy) coumarin.
1983	Gupta V.K.	Trunk bark of the *Aegle marmelos* by methanol extract.	studied coumarin compound R- (+) – marmin.

1991.	Nema, D, and Srivastava, S K.	Stem bark of the A. marmelos.	The isolation and structure elucidation of new compound: marmesin – 1"- α -L - rhamnopyranoside and 1,5 - dihydroxy - 6 - methoxy -2 -methyl anthraquinone.
1994	Ohashi K.	Bark.	Isolation of two new 7-gerayloxycoumarins and aeglins.
1995	Ohashi K.	Bark.	Isolation of four isomeric lignan-glucosides, two new lignan – glucoside, (-) – lyoniresinol 2α-O-β-D glucopyranoside and (-) 4 - epi-lyoniresinol, 3α -O -β -D-glucopyranoside, two known lignan - glucosides, (+)- lyoniresinol. 3α-O-β-D-glucopyranoside and (-)-lyoniresinol 3α-O-β-D-D-glucopyranoside
2000	Riyanto, S S, and Mustafa, A L. Majalah	Bark from Petroleum ether extract	Isolation of two terpenes, lupenone and lupeol.
2004	Samaraseke ra, I K R, and Krishnamurti , J K.	Various part	Isolation of various coumarin, Umbeliferone, Skimmin, Impertonin
April, 2004	Muhammad Shaiq Ali	Leaves of methanolic	Isolation of :7-geranyloxycoumarin or marmenol, praealtin D, *trans*-

	and Muhammad Kashif Pervez of H.E.J.	extract	cinnamic acid, valencic acid, 4-methoxy benzoic acid, betulinic acid, N-*p-cis*- and *trans*-coumaroyltyramine, montanine, and rutaretin.
November 2, 2007.	K. Sudharameshwari and J. Radhika.	Chloroform extract.	Shows presence of alkaloids, carboxylic acids, saponins and xanthoproteins.
November 2, 2007.	K. Sudharameshwari and J. Radhika	Petroleum ether extract.	Shows presence of flavonoids, anthocyanin.
December 20, 2007.	Nishi Sondhi , Renu Bhardwaj , Satwinderjeet Kaur, Neeraj Kumar and Bikram Singh.	Leaves	Isolation of 24-epibrassinolide.
August 14, 2008.	Preecha Phuwapraisirisan and his coworkers.	Leaves	Reported presence of phenylethyl cinnamides, anhydromarmeline, aegelinosides A and B.
December 3, 2008.	Shaheen Faizi ,	Fresh leaves	Reported alkaloid, shahidine as a major constituent.

	Fatima Farooqi and their coworkers.		

Table 03: Pharmacological research findings

Time Chronicles	**Name of the researchers**	**Part of the plant**	**Findings**
1981	Singh, K P, Chaturvadi, G N.	Pulp of *A. marmelos.*	Determination of antiprotozoal activity in chronic dysentery
1997	Rana, B K, and Jain, A K.	Leaves	Proved anti-fungal activity of essential oil isolated from the plant.
2000	Aritajat, S, Kaweewat, K, Manosroi, J. and Manosroi, A.	Aqueous extract of whole plant	No toxic effect on male rat reproduction and progeny outcome.
2001	Shoba, F G, and Thomas, M.	Unripe fruit	The methanolic plant extracts were more effective than aqueous plant extracts against castor-oil induced diarrhoea.
28 May, 2003.	N. Kamalakkannan and P. Stanely Mainzen Prince	Water extract of the fruits	Proved the hypoglycaemic effect of the fruit extract.

2003	Dhuley, J N.	Unripe fruit extract.	a possible antidiarrhoeal effect of unripe fruit extract of *A. marmelos*
October 22, 2004,	Ganesh Chandra Jagetia, Ponemone Venkatesh and Manjeshwar Shrinath Baliga.	Hydroalcoholic extract.	Found antitumor effect
2004	Upadhya, S., Shanbhag. K K, Suneetha, G, Naidu, B M. and Upadhya, S.	Aqueous extract of *Aegle marmelos* leaves	Owing to hypoglycemic and antioxidant properties, *Aegle marmelos* leaves may be useful in the long-term management of diabetes.
2005	Jagetia, G C, Venkatesh, P, and Baliga, M S.	Fruit extract	Significant reduction of the deleterious effect of radiation in the intestine and bone marrow of mouse.
2005	Latica, V, and Costa, L.	Extracts of Aegle marmelos	Evaluation of anticancer potential using brine shrimp lethality assay. Showed toxicity.
November 12, 2005.	Meera Sumanth and Mustafa	Ethanolic extract of unripe fruits	Found significant antistress, adoptogenic activity by improving the swim duration and reducing the elevated WBC, blood glucose and

			plasma cartisone.
2006	Citarasu, T, Sivaram, V, Immanuel, G, Rout, N, and Murugan, V.	Methanolic extract	Influence of selected Indian immunostimulant herbs against white spot syndrome virus (WSSV).
2006	Das U K.	A. marmelos	Determination of Testicular Steriogenic Enzyme.
2007	Miyazaki, S, Ranganatha n, D, and Kadarkarisw ami, M.	total alcoholic, total aqueous, whole aqueous and methanolic extracts isolated from the leaves	Elucidation of toxicity of the *A. marmelos*. Wide therapeutic window and High therapeutic index was found.
2008	Dhalwal K, Shinde V. M ., Namdeo A. G.,	Methanol extract of fruit pulp.	Antioxidant Profile and HPTLC-Densitometric Analysis of Umbelliferone and Psoralen.
On august 14, 2008,	Preecha Phuwapraisir isan	Leaves	Found a new series of α-glucosidase inhibitors.
March 3, 2009.	Vijaya C, Ramanathan M, Suresh B	Ethanolic extract	Proved the Lipid lowering activity in hyperlipidaemic models of Wistar albino rats.

Table 04: Antimicrobial studies on *Aegle marmelos*

Time Chronicles	Name of the researchers	Part of the plant	Findings
1981	Singh, K P, Chaturvadi, G N.	The pulp of A. marmelos.	Determination of antiprotozoal activity in chronic dysentery
April, 2004	Radhika Samaraseke ra J.K.R, Bhupinder P. S. Khambay; K. Patrick Hemalal.	Fractionation of the ethyl acetate extracts of the stem bark	A new insecticidal protolimonoid from *Aegle Marmelos* was discovered.
November 2, 2007.	Sudharames hwari K., Radhika J.	Extracts of *Aegle marmelos* petroleum ether, ethanol, chloroform and aqueous as solvent.	The maximum antibacterial efficiency was found to be present in petroleum ether extract of *Aegle marmelos*.

From the above tables, we can conclude that various parts of Aegle marmelos have shown to be therapeutically effective as well as a rich source of possible newer chemicals. It also showed some antibacterial properties. Therefore further research in this pursuit, focusing on the isolation of individual compounds and finally subjecting to

clinical trails promises to open new avenues in the use of this plant for therapeutic purpose.

Pharmaclogical Experiment

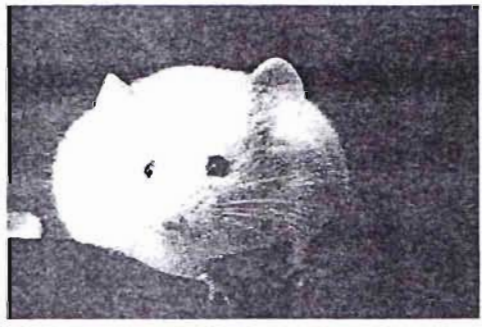


Fig 08: A swiss albino mouse

Tests for Antinociceptive Effect of A. marmelos

Collection and Identification of Aegle marmelos

About 2 kg of the Aegle marmelos bark has been collected from Ramergao, a village in the district Munshigonj. Identification of this plant part has been ensured by Bangladesh National Herbarium (DACB Accession number 34358).

Preparation

For this writhing test, we required to make four groups, each comprising of six mice. Therefore we took 16 mice altogether. Each of those weighed within 18 to 30 gm. Those were kept and observed in plastic cages having dimension of (28 X 22 X 13) cm. To prepare the mice beds soft wood shavings with pellets of mice foods were provided to the cages. The pellets were prepared by ICDDR, B (Animal Research Branch of the International Centre for Diarrhoeal Diseases and Research, Bangladesh). The experiment was designed to determine the analgesic activity of methanolic extract of the bark of *Aegle marmelos*. For preparing extract about 100 gm of bark dust was soaked with 300 ml of methanol for 2 days. Then the liquor was filtered and compounds dissolved in methanol solvent were separated. Then solvent was evaporated with the Rotary Evaporator and the extract was ready to be used. (Hossein, 2002).

Designing the Groups

16 experimental mice were randomly selected and divided into 4 groups denoted as-

Group A (Control)

Group B (Positive control)

Group C (Sample, 250mg/kg)

Group D (Sample, 500 mg/kg).

Mice Identification and Dosing

Several methods are particularly well suited for identifying cage mates. A felt tip marker may be used for marking an ear or tail. Such marks usually disappear in 1-2 days. We marked each of the mice in a group at a sequence of 1 to 6 before we started our experiment. Each mouse was weighed properly and the dose of the sample and control materials was adjusted accordingly.

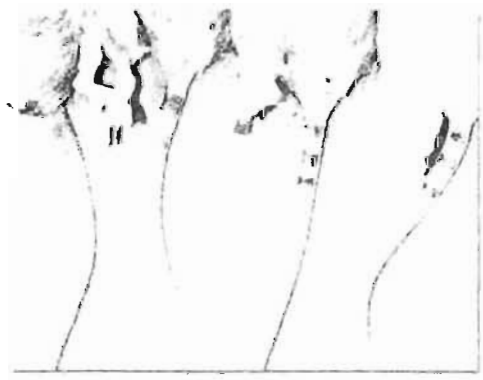


Fig 09: Mice identification by tail marking

Preparation of Sample Suspension

For 250 mg/kg body weight of the test sample

In order to receive a 250 mg of sample/kg body weight, 50 mg of sample was taken. The extract was triturated unidirectly by addition of small amount Tween-80. After

proper mixing, distilled water was slowly added. Therefore our sample suspension was prepared.

For 500 mg/kg body weight of the test sample

In order to receive a 500 mg of sample/kg body weight, 100 mg or 0.1 gm of sample was taken. The extract was triturated unidirectly by addition of small amount Tween-80. After proper mixing, distilled water was slowly added. Therefore our sample suspension was prepared.

Preparation of Negative and Positive Control

0.9 % NaCl solution was prepared to be administered intraperitoneally. 0.9 ml NaCl solution was taken from the 100% NaCl solution and then was diluted with 99.1 ml sterile water. According to a standard procedure 10 mg/kg i.p. Diclophenac was also prepared from Clofenac IM inj. (Each 3 ml contains 75 mg Diclofenac sodium BP). (Hossein H., 2002).

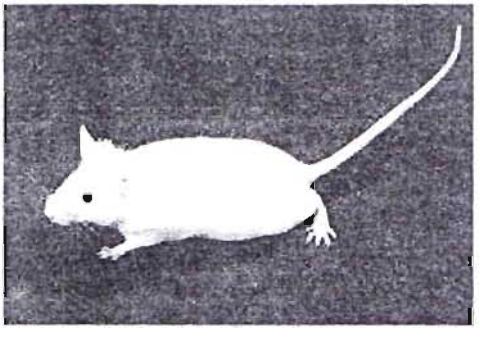


Fig 10: A mouse weighing within 20-30 gm

Procedure

Control (0.9 % NaCl solution) and Diclofenac sod. were given intraperitoneally by means of a 100 cc or 1 ml insulin syringe.

Test samples (250 mg/kg and 500 mg/kg) were given orally by means of a long needle with ball-shaped end to the mice.

A 30 minutes interval was given to ensure absorption. For group C and D, one hour interval after the administration of the extract was given.

Then each of the mice of groups was given intraperitoneal injection of 0.6% v/v acetic acid solution (volume of injection 0.1ml/10gm). Since acetic acid is the writhing inducing chemical, it will create nociception to the animal.

After an interval of 5 minutes, which was given for absorption of acetic acid, number of squirms or writhing was counted for 20 minutes. The number of writhes produced in these animals was counted for 30 minutes. For scoring purposes, a writhe is indicated by stretching of the abdomen with simultaneous stretching of at least one hind limb. Control received normal saline (10ml/kg i.p) and diclofenac (10mg/kg i.p) was used as a reference drug.

Observation with the Negative Group:

Since 0.9% normal saline does not have any effect on the reduction of pain, therefore it was used as a negative control. Mice of the group A had received this control. The

Observation with the Negative Control Group has been summarized in the following table.

Table 05: writhing count with the mice of negative control group

No. of mice	Body weight (gm)	Negative control administration time	Acetic Acid administration time	Writhing number	Mean writhing
1	20.1	1:21 pm	1:53 pm	19	18.8333
2	19.5	1:22 pm	1:55 pm	12	
3	25.9	1:23 pm	1:58 pm	17	
4	24.5	1:24 pm	1:51 pm	19	
5	19.1	1:26 pm	2:01 pm	28	
6	20.6	1:27 pm	2:04 pm	18	

Observation with Positive Control

Usually positive control group refers to a group of test animals which will have a reference drug which is capable to produce the desired activity that researchers want to check. Reference drug can be a standard drug or an effective herbal medicine or any kind of compound (either organic or inorganic), but it must possess a proven activity. For this test, we used Diclofenac (10 mg/kg). This was given intraperitoneally by means of a 100 cc or 1 ml insulin syringe to the group B. The Observation with the Positive Control Group has been summarized in the following table.

Table 06: Writhing count with the mice of positive control group.

No. of mice	Body weight (gm)	Drug administration time	Acetic Acid administration time	Writhing number	Mean writhing
1	18.9	2:10 pm	2:40 pm	1	2.167
2	19.8	2:11 pm	2:42 pm	6	
3	18.3	2:13 pm	2:43 pm	0	
4	23.4	2:15 pm	2:45 pm	1	
5	25	2:16 pm	2:48 pm	3	
6	24.6	2:17 pm	2:50 pm	2	

For Methanolic Extract of *Aegle marmelos* bark (250 mg/Kg)

The methanolic extract of *A. marmelos* bark part was administered orally to the Group C. The dose was 250 mg/kg. Observation with the Group C has been summarized in the following table.

Table 07: Writhing count with the mice of group (receiving 250 mg/Kg of the extract)

No. of mice	Body weight (gm)	Drug administration time	Acetic Acid administration time	Writhing number	Mean writhing
1	17.4	1:50 pm	2:20 pm	0	4.917
2	26	1:53 pm	2:24 pm	5	
3	24.5	1:55 pm	2:25 pm	0	
4	25.2	2:00 pm	2:30 pm	10.5	
5	28.1	2:03 pm	2:35 pm	1	
6	28	2:06 pm	2:38 pm	13	

Statistical Evaluation of the Activity of Methanolic Extract of *Aegle marmelos* Bark

We can make this evaluation by comparing analgesic activity of sample and control group. For this a hypothesis was built stating –

H_0 = There is no difference between the antinociceptive activity of the control and AMBE 250 (*A. marmelos* bark extract 250 mg/kg). This is our null hypothesis. i.e $\mu_1 = \mu_2$

H_A = There is a significant difference in antinociceptive activity between the control and sample. i.e, $\mu_1 \neq \mu_2$

Table 08: Calculation of means

x_1	$(x_1 - x'_1)$	$(x_1 - x'_1)^2$	x_2	$x_2 - x'_2$	$(x_2 - x'_2)^2$
19	0.16667	0.027779	0	-4.91667	24.17361
12	-6.83333	46.6944	5	0.083333	0.006944
17	-1.83333	3.361099	0	-4.91667	24.17361
19	0.16667	0.027779	10.5	5.583333	31.17361
28	9.16667	84.02784	1	-3.91667	15.34028
18	-0.83333	0.694439	13	8.083333	65.34027
$\sum$= 113		$\sum$=134.8333	$\sum$= 29.5		$\sum$= 160.2083

Here,

x_1 = frequency of writhing of group A.

x_2 = frequency of writhing of group C.

x'_1 = mean of the x_1

x'_2 = mean of the x_2

s = Pooled standard deviation

n_1 and n_2 = number of observation

Calculated means from two groups:

$x'_1 = 113 / 6 = 18.83333$ $\quad\quad$ $x'_2 = 29.5 / 6 = 4.916667$

So the pooled standard deviation, s:

$$s = \sqrt{\{(n_1-1)\, s_1^2 + (n_2-1)\, s_2^2\} / (n_1 + n_2 - 2)}$$

$$s = \sqrt{\{\sum (x_1 - x'_1)^2 + \sum (x_2 - x'_2)^2\} / (n_1 + n_2 - 2)}$$

$$s = \sqrt{(134.83331 + 160.2083) / (12 - 2)}$$

$$= 1.717678$$

Calculation for t :

$$t = (x'_1 - x'_2) / s \sqrt{(1/n_1 + 1/n_2)}$$

$$= (18.83333 - 4.916667) / 1.717678 \sqrt{(1/6 + 1/6)}$$

$$= (13.416663) / (1.717678 \times 0.577350)$$

$$= 13.416663 / 0.99170$$

= 13.52895

Calculation for degree of freedom

$n_1 + n_2 - 2 = 6 + 6 - 2 = 10$

Significance level, $\alpha = 5\%$ (2.228)

Interpretation :

The calculated value (13.52895) i.e (13.53) is greater than the table value (2.23) i. e , $p < 0.05$. Since $p < 0.05$ (According to t test calculator, p=0.00), the null hypothesis is rejected. Hence we conclude that there is a significant difference in the efficacy of the control and ABME 250 mg / kg in case of reducing pain. Therefore Alternative hypothesis $\mu_1 \neq \mu_2$ has been accepted.

Confidence interval :

Lower limit

$$(x_1' - x_2') - t_{2.5} (s \sqrt{(1/n_1 + 1/n_2)}$$

= (18.83333 – 4.916667) – 2.23 x .99170

=13.416663 – 2.211491

= 11.205172

Upper limit

$$(x_1' - x_2') + t_{2.5} (s \sqrt{(1/n_1 + 1/n_2)}$$

=(18.83333 – 4.916667) + 2.23 x .99170

=13.416663 + 2.211491

= 15.628154

Therefore, 95% confidence interval for the difference between two population means will be (11.205, 15.628).

Interpretation

The confidence interval does not include 0. Hence we can reject H_0 and conclude that there is significance difference between the two means at 5% level of significance.

For Methanolic Extract of *Aegle marmelos* bark (500 mg/Kg):

The methanolic extract of *A. marmelos* bark part was administered orally to the Group D. The dose was 500 mg/kg. Observation with the Group D has been summarized in the following table.

Table 09: Writhing count with the mice of group D (receiving 500 mg/Kg of the extract)

No. of mice	Body weight (gm)	Drug administration time	Acetic Acid administration time	Writhing number	Mean writhing
1	25	11:46 am	12:20 pm	0	0.5
2	24.8	11:50 am	12:22 pm	0	
3	22	11:53 am	12:24 pm	3	
4	23.1	11:56 am	12:27 pm	0	
5	15.4	11:59 am	12:30 pm	0	
6	26.8	12:01 am	12:33 pm	0	

Statistical Evaluation of the Activity of Methanolic Extract of *Aegle marmelos* Bark (500mg/kg)

We can make this evaluation by comparing analgesic activity of sample and control group. For this a hypothesis was built stating –

H_o = There is no difference between the antinociceptive activity of the control and AMBE 500 (*A. marmelos* bark extract 500 mg/kg). This is our null hypothesis. i.e $\mu_1 = \mu_2$

H_A = There is a significant difference in antinociceptive activity between the control and sample. i.e, $\mu_1 \neq \mu_2$

Table 10: Calculation of means

x_1	$(x_1 - x'_1)$	$(x_1 - x'_1)^2$	x_2	$x_2 - x'_2$	$(x_2 - x'_2)^2$
19	0.16667	0.027779	0	-0.5	0.25
12	-6.83333	46.6944	0	-0.5	0.25
17	-1.83333	3.361099	3	2.5	6.25
19	0.16667	0.027779	0	-0.5	0.25
28	9.16667	84.02784	0	-0.5	0.25
18	-0.83333	0.694439	0	-0.5	0.25
$\sum$ = 113			$\sum$ = 3		$\sum$ = 7.5

Here,

x_1 = frequency of writhing of group A.

x_2 = frequency of writhing of group D.

x'_1 = mean of the x_1

x'_2 = mean of the x_2

s = Pooled standard deviation

n_1 and n_2 = number of observation

Calculated means from two groups:

$x'_1 = 113 / 6 = 18.83333$ $x'_2 = 3 /6 = 0.5$

So the pooled standard deviation, s

$$s = \sqrt{\{(n_1-1)\, s_1^2 + (n_2-1)\, s_2^2\} / (n_1 + n_2 - 2)}$$

$$s = \sqrt{\{\sum (x_1 - x'_1)^2 + \sum (x_2 - x'_2)^2\} / (n_1 + n_2 - 2)}$$

$$s = \sqrt{(134.83331 + 7.5) / (12 - 2)}$$

$$= 3.77271$$

Calculation for, t.

$$t = (x'_1 - x'_2) / s\sqrt{(1/n_1 + 1/n_2)}$$

$$= (18.83333 - 0.5) / 3.77271 \sqrt{(1/6 + 1/6)}$$

$$= (18.33333) / (3.77271 \times 0.577350)$$

$$= 18.33333 / 2.178175$$

$$= 8.416830$$

Calculation for degree of freedom

$n_1 + n_2 - 2 = 6 + 6 - 2 = 10$

Significance level $\alpha = 5\%$ (2.228)

Interpretation :

The calculated value (8.416830) i.e (8.42) is not less than the table value (2.23) i. e , $p < 0.05$. Since $p < 0.05$ (According to t test calculator, p=0.000008), the null hypothesis is rejected. Hence we conclude that there is a significant difference in the efficacy of the control and ABME 500 mg / kg in case of reducing pain. Therefore Alternative hypothesis $\mu_1 \neq \mu_2$ has been accepted.

Confidence interval

Lower limit

$(x_1' - x_2') - t_{25} (s \sqrt{(1/n_1 + 1/n_2)}$

$= 18.33333 - 2.23 \times 2.178175$

$= 13.47599$

Upper limit

$(x_1' - x_2') + t_{25} (s \sqrt{(1/n_1 + 1/n_2)}$

$= 18.33333 + 2.23 \times 2.178175$

$= 23.19066$

Therefore, 95% confidence interval for the difference between two population means will be (13.476, 23.191).

Interpretation

The confidence interval does not include 0. hence we can reject H_0 and conclude that there is significance difference between the two means at 5% level of significance.

Statistical Analysis of the Results Shown:

Table 11: Showing the statistical analysis of the results from four test groups.

Animal group	Mean writhing	% writhing	% inhibition	SD	SE	SE for % writhing
Negative control	18.8333	100	0	5.1928	2.11995	11.25639
Positive control	2.1667	11.50639	88.49361	2.13697	0.87241	4.63227
AMBE 250 mg	4.9167	26.10641	73.89358	5.43937	2.22061	11.79087
AMBE 500 mg	0.5000	2.65487	97.34512	1.11803	0.45643	2.42352

Here, AMBE stands for *Aegle marmelos* bark extract.

Another way of showing the comparative data analysis is given below:

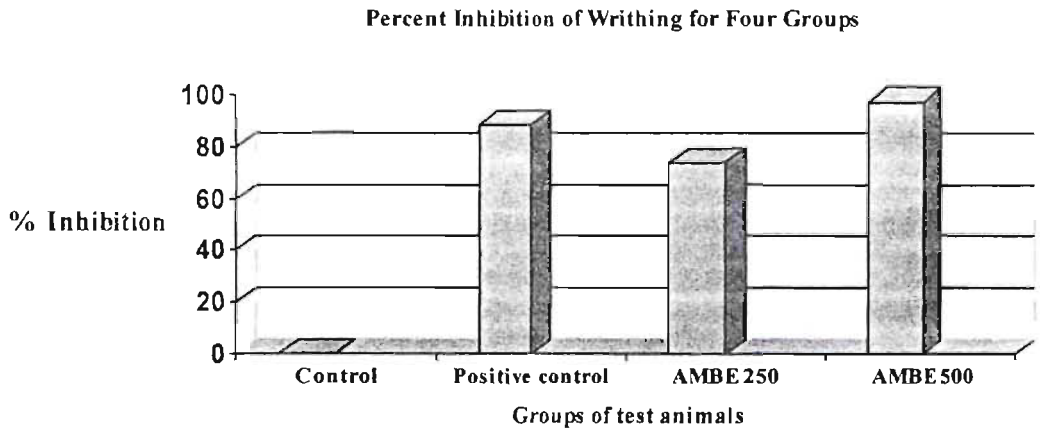


Fig 11: Comparison between four different test animal groups in terms of the percent writhing inhibition.

Result

The percent inhibition of writhing observed by the bark extract administered at a dose of 250 mg/kg was 73.89% and that for the dose of 500 mg/kg was 97.34%; positive control Diclofenac showed percent inhibition of writhing of about 88.49% while this data for negative control is considered as 0%. The statistical value indicates that for methanolic extract of *Aegle marmelos* 250 mg/kg, calculated t value and p value were 13.52 and 0.00 respectively and for 500 mg/kg, t value 8.42 and p value was 0.00008.

Conclusion

All data clearly showed that the methanolic bark extracts of *Aegle marmelos* can exert pain relieving or antinoceiceptive activity. Both the extracts (250 mg/kg and 500 mg/kg) effectively reduced writhing frequency in mice. The analgesic activity increases as the dose of the sample increases.

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