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RESEARCH ARTICLE

COMPARATIVE PHYTOCHEMICAL EVALUATION AND ANTIMICROBIAL ACTIVITY STUDIES ON MEDICINAL TREE AEGLE MARMELOS (L.) CORREA AND ITS INTRASPECIFIC VARIANT "EKABILVA"

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Abstract

The present study is focused on the comparative phytochemical analysis and antimicrobial activity in the typical form of the Aegle marmelos(AM1) and its intraspecific variant EkaBilva(AM2). Since ancient times "Bilva"is highly used in the indigenous systems of medicinesfor various disorders but extensively for dysentery and diabetes.Its intraspecific variant "EkaBilva"has more extensively used rather than "Bilva" by the locals in Telugu speaking states. To find out the variations in the phytochemical components and their activity in the both typical form and intraspecific variant the studies were carried out. The powdered leaf materials of both were extracted by using Aqueous, ethanol, chloroform and pet-ether extracts. The following chemical compounds were reported Alkaloids, Steroids, Triterpenoids,Tannins, Glycosides, Flavonoids, Reducing sugars, Phenolsin AM1&AM2. Tannins, Flavonoids are present in Alcohol extracts in AM1 differing with AM2. The antimicrobial activity was carried out by disk-diffusion method by using four different microorganisms with the nutrient agar medium the high zone of inhibition shown in the leaf extracts of AM2 in all extracts except aqueous in all isolates.

Introduction:-

The genus **Aegle** Correa belongs to family Rutaceae, represented with only two species **Aegle decandra** Fern-Vill. (the native range of the species is Philippines) and **Aegle marmelos**(L.) Correa (the native range of the species is Indian subcontinent – Bangladesh, India, Nepal, Pakistan, Western Himalayas)(POWO, 2024). **A. marmelos**commonly known as Bale tree or Bilva treein India and it grows from dry deciduous forests to the wet evergreen forestsalmost throughout the country. Also, it is very abundant outside forests on the hedges of agricultural fields, along roadsides, open lands etc. For its immense health benefits and its sacred importance,it is widely introduced in cultivation and plantations near temples science prehistoric times ca. 5000 years mentioned in the ancient ayurvedic text in CharakaSamhitha (Kirtikar and Basu1991, ChamilakP et al., 2020).Due to its adaptation in varied climatic and geographical areas the species exhibit morphological variations(intraspecific variations) in the structure of foliage, presence and absence of thorns, size and shape of the fruit. The Botanists/Taxonomists based on the variations they described as varieties (infraspecific taxa) A. marmelos var. mahurensis, A. marmelos var. tamilnadensis.But laterGovaerts (1995) synonymized the both the varieties under typical form of the species **A. marmelos.**The intraspecific variants taxonomically stand as a

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infraspecific taxa or not but in local and traditional medicine they have demand for their effective relief from the various disorders rather than the typical form of the species (Prashith 2014,Sandhya et al., 2024,). "EkaBilva" is one of the intraspecific variants in the **A. marmelos**, found growing in Andhra Pradesh and Telangana states and also planted large scales near temples for its sacred importance (one leaflet bilva- Ekabilva). The Ekabilva intraspecific variant of the **A. marmelos**, which is differing from the typical form of the species by the following: Large canopy, absence of thorns, leaf let lamina partially lobed to deeply three lobed (appears like simple leaf), inflorescence in clusters, size of the fruit is very small. The studies pertaining to the phytochemical analysis in the various parts of the Bilva carried out for drug discovery (Chanda 2008, Nirupama 2012, Katiyar 2012) but not comparative with intraspecific variants. To find out the variations in the phytochemicals in in the typical form of the species **A. marmelos**and its intraspecific variant "EkaBilva" the present studies carried out.

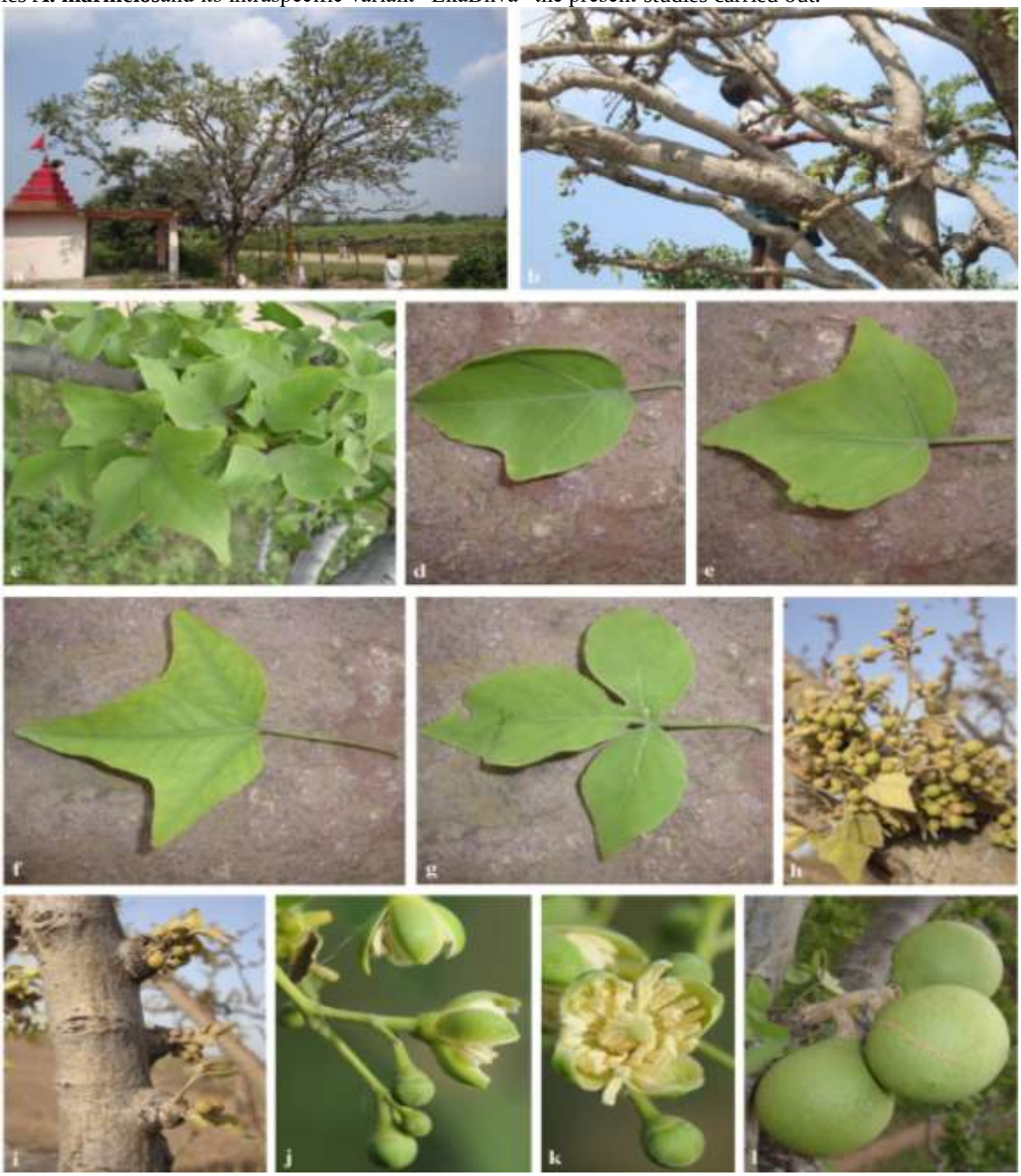

Figure 1:- Aegle marmelos – Intraspecific variant "EkaBilva". Legend: a –Habit. B. Stems without thorns. c. Foliage. d - g. Leaf lamina partially lobed to three lobed in the single leaf cluster. h-i. Inflorescences. j – k. Flowers. l. Fruits.

Material and Methods:-

Plant material:

Foliage of A. marmelos(AM1) collected from the naturally growing plants and its intraspecific variant "EkaBilva"collected from the plantations/Cultivation near temples (Wild source unknown).The live images of "EkaBilva" (AM2) with details of habit, canopy, foliage variation, inflorescence, flowers and fruits given in the plate 1 for finding the morphological differences with typical form of the species A. marmelos(Bilva).

Preparation of extracts:

The fresh collected leaf material of the AM1 & AM2was made free from foreign particles. Later they segregated manually and took in a plastic traysand labelled as AM1 &AM2. Then both of them kept in a shade for drying. After complete dry of the leaf material (18 days) grinded by Blender Machine and coarse powder collected separately for two samples. The powdered leaf material (250 gm) was extracted with Aqueous, Ethanol, Chloroform and Pet-ether and water by cold extraction method. The extract was filtered through a fresh cotton bed and finally with Whatman no. 1 filters papers.

Preliminary Phytochemical Screening

Phytochemical screening was performed to identify phytochemicals in the Aqueous, Ethanol, Chloroform and Pet-ether extracts.

The phytochemicals were detected by colortests.

Test for alkaloids:

Of each extract 2ml was acidified with a few drops of dilute hydrochloric acid. Then 1ml of Dragendorff's reagent was added. The appearance of orange to red precipitate indicates the presence of alkaloids.

Test for tannins:

To 2ml of each extract a few drops of 10% lead acetate were added. The appearance of white precipitate indicates the presence of tannins.

Test for saponins:

To 1ml of extract taken in a measuring jar,9ml of distilled water was added and shaken vigorously for 15seconds and extract were allowed to stand for 10min.Formation of stable foam(1cm) indicates the presence of saponins.

Test for steroids:

Chloroform 10ml was added to 2ml of all three plant extracts. To these extracts 1ml of acetic anhydride was added and then 2ml of concentrated sulphuric acid was added along the sides of the test tube. Color formation at the junction is noted. The appearance of blue green color indicates the presence of steroids.

Test for Triterpenoids:

The test for Triterpenoids is same as that for steroids the appearance of red, pink color or violet color at the junction indicates the presence of Triterpenoids.

Test for glycosides:

To 1ml of each extract a few drops of glacial acetic acid and ferric chloride and 3-4 drops of concentration sulphuric acid were added. The appearance of blue-green color indicates the presence of glycosides.

Test for flavonoid:

4ml of extract solution was treated with 1.5ml of methanol solution. The solution was warmed and metal magnesium was added to this solution 5-6 drops of Con. HCl acid were added and colour was observed for flavonoids and orange colour for flavones.

Test for reducing sugar:

To 0.5ml of extract solution, 1ml of water and 5-8 drops of Fehling's solution was added to the test tube hot and observed for brick red precipitate.

Test for Resins:
10 mL of distilled water was added to extract, to which a few drops of 4% HCl were added. Appearance of turbidity in solution indicates presence of resins.

Test for Phenolic compounds:
Treat the extract with ferric chloride solution, blue color appears if hydrolysable tannins are present and green color appears if condensed tannins are present.

The phytochemical screening was performed in Aegle marmelos (Bilva) and its intraspecific variant (Ekabilva) leaf extracts of polar and non-polar solvents and the results are tabulated in Table-1 &2 respectively.

Table 1:- Showing phytochemical screening in leaf extracts of AM1(Bilva).

S.No.	PhytochemicalTests	Aqueous	Ethanol	Pet Ether	Chloroform
1	Alkaloids	+	+	-	-
2	Tannins	+	+	-	-
3	Saponins	-	-	-	-
4	Steroids	-	-	+	-
5	Triterpenoids	+	+	-	-
6	Glycosides	-	-	+	+
7	Flavonoids	+	+	-	-
8	Reducingsugar	+	+	-	-
9	Resins	-	-	-	-
10	Phenols	+	-	-	-

Table 2:- Showing phytochemical screening in leaf extracts of AM2 (EkaBilva).

S.No.	hytochemicalTests	Aqueous	Ethanol	Pet Ether	Chloroform
1	Alkaloids	+	+	-	-
2	Tannins	+	-	-	-
3	Saponins	-	-	-	-
4	Steroids	-	+	+	+
5	Triterpenoids	-	+	-	-
6	Glycosides	+	+	-	+
7	Flavonoids	+	-	-	-
8	Reducingsugar	+	+	-	-
9	Resins	-	-	-	-
10	Phenols	+	+	-	-

Antimicrobial Activity
Disc preparation:
The 6mm (diameter) discs were prepared from what man No.1 filter paper and the discs were sterilized by autoclave at 121^{O}C. After sterilization the moistened discs were kept in hot air oven at 50^{O}_{C}. Then disc were impregnated with suitable concentration of extracts from stock of50mg/ml.Fourdifferentmicroorganisms:E.coli(MTCC:41),P.aeruginosa(MTCC:424),B.cereus(MTCC:430)and B.subtilis(MTCC:441).Thesolventwithoutextracts served asnegative control. Standard antibiotic streptomycin(10µg),Ampicillin (10µg) wereemployed aspositive control.

Disk-Diffusion Method:
Antimicrobial activity was carried out by disk-diffusion method using nutrient Agar medium. 100 micro liter of suspension containing 10^{8} colony forming units mL-1 of bacteria spread over the nutrient agar medium plates by using separate sterile cotton buds. After the microbial lawn preparation extracts of plant discs (aqueous, ethanol and acetone extract) were firmly pressed on to the agar surface of each seeded plate. Petri dishes were incubated at 37^{0} C for 24 h and the average diameter of the inhibition zone surrounding the wells was determined visually.

Antimicrobial activities of the extracts were expressed by – (no zone of inhibition), + zone of inhibition=8mm in diameter (low zone of inhibition) and ++ zone of inhibition>8mm in diameter (moderate zone of inhibition), +++ zone of inhibition ≥12mm in diameter (high zone of inhibition). All the tests were performed in duplicate and repeated for confirmation of result.

Anti-microbial activity is performed in the leaf extracts of Aegle marmelosL. and its variety against four species of bacteria viz.Escherichia coli (MTCC:41), **Pseudomonas** aeruginosa (MTCC:424), Bacillus cereus (MTCC:430), Bacillus subtilis (MTCC:441). The results of zone of inhibition (in mm) per 100mg/ml and anti-microbial activity in various polar and non-polar solvents is explained in Table- 3 & 4 respectively. Similarly, zone of inhibition and anti-microbial activity of EkaBilva (AM2) are shown in Table-5 & 6. Photos are exhibited in plate No. 1 & 2

Table 3:- Showing zone of inhibition (in mm) of various leaf extracts of plant AM1.

Micro organism	Aqueous (100mg/ml)	Ethanol (100mg/ml)	Pet-ether (100mg/ml)	Chloroform (100mg/ml)
E.coli (MTCC:41)	**33.09 mm**	**8.01 mm**	**25.24mm**	**2.39mm**
P.aeruginosa(MTCC:424)	**1.76 mm**	**1.98 mm**	**8.35mm**	**18.01mm**
B.cereus(MTCC:430)	**0.96 mm**	**21.55 mm**	**32.62mm**	**55.48 mm**
B.subtilis(MTCC:441)	**14.63 mm**	**68.41 mm**	**18.26mm**	**5.44mm**

Table 4:- Showing Anti-microbial activity of various leaf extracts of plantAM1.

Micro organism	Aqueous	Ethanol	Pet-ether	Chloroform
E.coli (MTCC:41)	+++	+	+++	-
P.aeruginosa(MTCC:424)	-	-	+	+++
B.cereus(MTCC:430)	-	+++	+	+++
B.subtilis(MTCC:441)	+++	+++	+++	-

Antimicrobial activities of the extracts were expressed by – (no zone of inhibition), + zone of inhibition=8mm in diameter (low zone of inhibition) and ++ zone of inhibition>8mm in diameter (moderate zone of inhibition), +++ zone of inhibition ≥12mm in diameter (high zone of inhibition).

Plate 1:- Showing zone of inhibition of leaf extract ofAM1plant in various solvents.

Micro organism	Aqueous	Ethanol	Pet-ether	Chloroform
E.coli (MTCC:41)				
P.aeruginosa(MTCC:424)				

B.cereus (MTCC:430)				
B.subtilis(MTCC:441)				
				

Table 5:- Showing zone of inhibition (in mm) of various leaf extracts of plantAM2.

Micro organism	Aqueous (100mg/ml)	Ethanol (100mg/ml)	Pet-ether (100mg/ml)	Chloroform (100mg/ml)
E.coli (MTCC:41)	**10.80 mm**	**31.55 mm**	**2.82 mm**	**10.10 mm**
P.aeruginosa(MTCC:424)	**12.60 mm**	**7.67 mm**	**18.33 mm**	**5.38 mm**
B.cereus(MTCC:430)	**24.56 mm**	**28.01 mm**	**35.21 mm**	**1.08 mm**
B.subtilis(MTCC:441)	**15.4 mm**	**5.39 mm**	**24.61 mm**	**11.18 mm**

Table 6:- Showing Anti-microbial activity of various leaf extracts of plantAM2.

Micro organism	Aqueous	Ethanol	Pet-ether	Chloroform
E.coli (MTCC:41)	++	+++	-	++
P.aeruginosa(MTCC:424)	+++	+	+++	-
B.cereus(MTCC:430)	+++	+++	+++	-
B.subtilis(MTCC:441)	+++	-	+++	++

Plate 2:- Showing zone of inhibition of leaf extract of AM2 plant in various solvents.

Micro organism	Aqueous	Ethanol	Pet-ether	Chloroform
E.coli (MTCC:41)				

P.aeruginosa(MTCC:424)				
B.cereus (MTCC:430)				
B.subtilis(MTCC:441)				
				

Results and Discussions:-

The preliminary phytochemical analysis in AM1 &AM2 showed the variation in the presence of alkaloids, tannins, saponins, steroids, triterpenoids, glycosides, flavonoids, reducing sugars, phenols and complete absence of saponins and resins in the extracts of aqueous, ethanol, pet-ether and chloroform results shown in the Table 1. In AM1, Alkaloids were absent in Pet-ether and Chloroform extracts, tannins shown absent in the pet-ether and chloroform extract, steroids absent in all except pet-ether, triterpenoids shown absent in the pet-ether and chloroform extracts, glycosides were absent in alcohol and aqueous extracts, flavonoids shown absent in the extracts of pet-ether and chloroform, phenols were present only in aqueous extract. In AM2, tannins, flavonoids are absent in all extracts except aqueous. Alkaloids, reducing sugars and phenols were present only in alcohol and aqueous extracts. Steroids ae absent in aqueous extracts. Glycosides were absent in pet-ether extracts results shown in the table 2. The glycosides and steroid compounds shown presence in majority of the extracts of AM2.

The antibacterial activity was performed in the leaf extracts of AM1&AM2 against four species of bacteria (**E. coli, B. cereus, B. subtilis, P. aeruginosa**) results shown in the Table 3 &4 (AM1) 5 & 6 (AM2) and Figures 1 (AM1) &

2 (AM2). The antimicrobial activities were expressed in all extracts against all isolates in leaf extracts of AM1 except in chloroform(**E.coli**),ethanol(**P.aeruginosa**),aqueous(**P.aeruginosa&B.cereus**))chloroform(**B.subtilis**) whereas in AM2 antimicrobial activity is shown in pet-ether against all and also chloroform shows against all except bacteria **E.coli**. The high zone of inhibition shown in the leaf extracts of AM2 in all extracts except aqueous in all isolates.

Conclusions:-

The comparative phytochemical studies in **Aegle marmelos** (Bilva-AM1) and its intraspecific variant (Eka Bilva-AM2) reveals the presence of more amount of glycosides, steroids and phenolic compounds in the leaf material of intraspecific variant (EkaBilva). This could be the reason for the leaf material of intraspecific variant "EkaBilva" isworking effectively and widely used as an antioxidant, antidiabetic and antimicrobial in traditional and local medicine. Further, the studies supporting and providing the scientific evidence for the local knowledge and beliefs intraspecific variant medicinal plants has more effectively work rather than the typical form of the species in general.

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