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PHYTOCHEMICAL SCREENING AND ANTI ALZHIEMER ACTIVITY FOR THE PEELS OF C*ITRUS MAXIMA*

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Abstract:

The literature survey showed that, only scrappy information was available on this shrub. With this scanty information on this plant consistent expectation of unexplored Phyto chemical profile and pharmacological efficacy under Rutaceae family forms the rationale for the study. Extraction and phytochemical evaluation of them deals with chemical analysis of the extract used for pharmacological screening. Qualitative preliminary phytochemical analysis was performed to detect the phytoconstituents nature and their presence in powder and its various extracts. Ethyl acetate extract showed the presence of steroids, flavanoids, tannins, triterpenoid and phenolic compound. Whereas the ethanolic extract showed the presence of steroids, flavanoids, phenols, phytosterol, gums and mucilage, carbohydrate, triterpenoids, volatile oil. Aqueous extract showed the presence of steroids, flavanoids, phenols, gums and mucilage, carbohydrate and triterpenoid. Based on the acute toxicity studies, previously reported 1/10th and 1/5th (200 and 400 mg/kg) of the maximum tolerated dose (2000 mg/kg B.W) were selected for the in vivo studies. The ethanolic extract of the peels of Citrus maxima was administered orally for fourteen days showed a dose dependent and significant improvement in memory of young mice and it also successfully reversed the memory deficits induced by Scopalamine. Furthermore a significant decreased in cholinergic transmission level in mice brain accounts for its multifarious beneficial effects such as anti alzheimer activity. Ethanolic extract showed the significant anti Alzheimer activity thus it was packed into a column to scrutinize the phytoconstituents present in it.

Key words: Phytochemical, Anti Alzhiemer Activity, Peels Of Citrus Maxima

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INTRODUCTION:

Alzheimer's Disease (AD) is a progressive neurodegenerative disease characterized by the loss of function of neurons and their consequent death [1]. Although AD is not a natural consequence of aging, it is generally seen in the elderly population [2]. The main symptoms encountered in Alzheimer's disease and Alzheimer's-related dementia are called neuropsychiatric symptoms (NPS) [2]. Although depression takes the first place, schizophreniform or paranoid psychosis is one of the other most common types of these symptoms [3]. Describing overt syndromes in patients can become quite complex, as these NPSs may overlap into clusters of symptoms [2]. However, some of the AD syndromes have been found to be quite consistent in AD in systematic studies performed on various populations [4,5]. These common syndromes include mild onset but progressive cognitive dysfunction, apathy, sleep disorders, psychosis, and agitation [6]. Alzheimer's patients also face many problems such as perception disorders, problems in learning skills and memory, slips of the tongue, executive dysfunction, regression in multitasking skills, and loss of confidence. Depending on the severity of dementia seen in patients, individuals become dependent on special care.

Several different diagnostic criteria have been published, examining cerebrospinal fluid biomarkers and imaging techniques for the classification of AD [7]. According to the Mini-Mental State Examination score (a short screening tool that expresses the severity of the condition in neurological disorders such as AD and dementia by grading cognitive impairment) [8] and the severity of the disease sequelae, AD can be examined in subgroups as moderate or severe [9]. In addition, although there are different opinions on the subject, the term pre-clinical AD is generally used for cases with AD pathology but no dementia, regardless of whether it has passed into the symptomatic stage [10]. Deterioration in memory function and difficulties in daily activities could occur in the advanced stages of AD progression, as well as they may start long before physical symptoms [11,12].

As in many neurodegenerative or chronic diseases, it is necessary to have knowledge about the role of certain individual differences such as gender, age, and race in susceptibility to the disease in AD. Based on this information, the creation of new therapeutic strategies to prevent disease will be more consistent, reliable, and faster [13,14,15]. Most of the plants are used by validating the traditional claims. There are various plants yet to be verified. *Citrus maxima* is one such fascinating shrub of family Rutaceae. It is commonly known as Papanus, Pommelo, shaddock, etc. The plant has a long history of numerous traditional and ethno botanical applications in diverse cultures. Many tribes considered it as a cure for all ailments. In the present study an attempt was made To evaluate the anti- Alzheimer activity of the peels of the fruit *Citrus maxima* using Morris water maze method and Radial Y maze

MATERIALS AND METHODS:

Plant Collection and Authentication

Fresh fruits were collected from Hyderabad, Telangana, authenticated. The Peels of fruit *Citrus maxima* were separated and shade dried, cut into pieces, powdered and was stored in the air tight container.

PHYTOCHEMICAL STUDIES

PRILIMNARY PHYTOCHEMICAL SCREENING

Phytochemical evaluation is used to determine the nature of phyto constituents present in the plant by using suitable chemical tests. Since the phytochemical constituents are responsible for the pharmacological activities of the plant. This study is essential to identify the constituents. Therefore a complete investigation is required to characterize the Phyto constituents qualitatively and quantitatively.

Preparation of Extracts

Extraction is the preliminary step involved in the phytochemical studies. It brings out the metabolites into the extracting solvent depends upon its polarity.

Extraction

The first step was the preparation of successive solvent extracts. The dried coarsely powdered sample of *Citrus maxima*. (J. Burm.) Merr peels (500gm) was first extracted with n- Hexane (60-80°C) in Soxhlet apparatus and then with solvents of increasing polarity like ethyl acetate and ethanol at 60 - 70ºC. They were then followed with maceration with chloroform water. Each extract was concentrated using rotary vacuum evaporator. The percentage yield, colour and consistency of these extracts were recorded and preceded for further detailed phyto chemical and pharmacological screening.

PRELIMINARY PHYTOCHEMICAL SCREENING

The chemical tests for various Phyto constituents in the dried powder and extracts of peels of *Citrus maxima*. (J. Burm.) Merr. were carried out as described below and the results were recorded.

1. **Detection of Alkaloids**

   Dragendorff's reagent :

   The substance was dissolved in 5ml of distilled water, to this 5ml of 2MHcL was added until an acid reactions occurs, then 1ml of Dragendorff's reagentwas added and examined for an immediate formation of an orange red precipitate. Mayer's reagent:

   The substance was mixed with little amount of dilute hydrochloric acid and Mayer's reagent and examined for the formation of white precipitate.

Wagner's reagent :
The test solution was mixed with Wagner's reagent and examined for theformation of reddish brown precipitate.

2. **Detection of Glycosides**

Borntrager's test :
The powdered material was boiled with 1ml of sulphuric acid in a test tube for five minutes. Filtered while hot, cooled and shaken with equal volume of chloroform. The lower layer of solvent was separated and shaken with half of its volume of dilute ammonia. A rose pink to red colour is produced in the ammonical layer.
Modified Borntrager's test :
The test material was boiled with 2ml of the dilute sulphuric acid. This was treated with 2ml of 5% aqueous ferric chloride solution (freshly prepared) for 5 minutes, and shaken with equal volume of chloroform. The lower layer of solvent was separated and shaken with half of its volume of dilute ammonia. A rose pink to red colour is produced in the ammonical layer.

3. **Detection of Steroids and Triterpenoids**
Libermann Burchards Test:
The powdered drug was treated with few drops of acetic anhydride, boiled and cooled. Conc.sulphuric acid was added from the sides of the test tube, brown ring is formed at the junction of two layers and upper layer turns green which shows presence of steroids and formation of deep red color indicates presence of triterpenoids.
Salkowski Test :
The extract was treated with few drops of concentrated sulphuric acid, red color at lower layer indicates presence of steroids and formation of yellow colored lower layer indicates presence of tri terpenoids.
Sulfur powder test:
Small quantity of test solution add sulfur powder. Sulpur powder sinks at the bottom shows the presence of triterpenoid.
Nollers test:
Test was mixed with tin and thionyl chloride. It produces pink colour whichindicates the presence of triterpenoids.

4. **Detection of Flavonoids**
Shinoda test :
To the solution of extract, few piece of magnesium turnings and concentrated HCl was added drop wise, pink to crimson red, occasionally green to blue color appears after few minutes indicates the presence of flavonoids.

Alkaline reagent test :
To the test solution few drops of sodium hydroxide solution was added, intense yellow color is formed which turns to colorless on addition of few drops of dilute acid indicate presence of flavonoids.
Zinc Hydrochloride test:
Small quantity of extract add a mixture zinc dust and Conc HCl producesred colour. It indicates the presence of flavonoids.

5. **Detection of Carbohydrates**
Molisch's test :
To the test solution few drops of alcoholic alpha napthol and few drops of conc. sulphuric acid were added through the sides of test tube, purple to violet color ring appears at junction.
Fehling's test :
The test solution was mixed with Fehling's I and II and heated andexamined for the appearance of red coloration for the presence of sugar.

6. **Detection of Phenols**
Ferric chloride test :
A small quantity of substance was dissolved with 2ml distilled water and a few drops of 10% aqueous ferric chloride solution was added and observed for appearance of blue or green color.

7. **Detection of Proteins**
Biuret test :
The sample was treated with 5-8 drops of 10% w/w copper sulphate and sodium hydroxide solution, violet color is formed.

8. **Detection of Tannins**
Lead acetate test :
The test solution was mixed with basic lead acetate solution and examined for formation of a white precipitate.
Ferric chloride test :
A few drops of 5% aqueous ferric chloride solution was added to 2ml of an aqueous extract of the drug and examined for the appearance of bluish black color.

9. **Detection of Saponins**
A drop of sodium bicarbonate solution was added to the sample and the mixture was shaken vigorously and left for 3 minutes. Development of any honey comb like froth was examined.

10. **Detection of Gums and Mucilage**
Small quantities of test substances was dissolved in 5 to 10ml of acetic anhydride by means of heat, cooled and add 0.05ml of concentrated sulphuric acid; it is examined for the formation of bright purplish red color.

11. **Detection of fixed oils and fats:**
Small quantities of extracts were pressed between two filter papers. Anoily stain on filter paper indicates the presence of fixed oils and fats.

PHARMACOLOGICAL STUDIES:
In *in-vitro* method, the ethanolic extract showed potent anti oxidant activity and acetyl cholinesterase inhibitory activity which was comparable with the standard than other extracts. Hence Ethanolic extract was selected for *in-vivo* study.

ACUTE ORAL TOXICITY STUDY (FIXED DOSE PROCEDURE) OECD 420 GUIDLINE FOR THE TESTING OF CHEMICALS[141-142]
The organization of economic co – operation and

development (OECD) guideline 425 was followed the acute oral toxic fixed dose method is a stepwise procedure with 5 rats of single sex per step (one animal per step). Depending upon the mortality and morbidity status of the animal, on average of 2 to 4 steps may be necessary to allow judgment on the acute oral toxicity of the substance. This procedure results in the use of minimal number of animal while allowing for acceptable data based scientific conclusion.

Literature survey showed that acute toxicity of the extracts was determined according to the OECD guideline No. 420 (20). Male albino mice weighing 27-30 g were used for Methanolic Extract of *Citrus maxima* was given to four groups (n = 5) of animals each at 5, 50, 300 and 2000 mg kg-1 b.w. p. o. The treated animals were under observation for 14 days, for mortality and general behavior. No death was observed till the end of the study. The test sample was found to be safe up to the dose of 2000 mg/kg. So, $1/10^{th}$ and $1/5^{th}$ of the dose (200 and 400mg/kg) were selected for this study.

***IN VIVO* EVALUATION OF ANTI ALZHEIMER ACTIVITYEXPERIMENTAL DESIGN:**

Materials and MethodsPlant extract

Ethanolic extract of seeds of *Citrus maxima.,* Burm..

***IN VIVO* EVALUATION OF ANTI ALZHEIMER ACTIVITY**

Scopalamine induced memory impairment[143]

The reversible cholinergic receptor antagonist scopolamine induces memory loss by impairing memory acquisition posses of short term memory in rodents and humans. Scopolamine also interferes in CNS cholinergic neurotransmission resulting in memory impairment.

Grouping of animals

Animals were divided into 4 groups of six animals each.

Group I: Scopalamine (0.3mg/kg b.wt) was injected after training. TL(Transfer Laterncy) was recorded after 1 hour of injection.

Group II: Rivastigmine (0.3 mg/kg b.wt) was injected for 14 days and on the 14^{th} day after 30min of drug administration, scopolamine (0.3mg/kg b.wt) was given i.p. TL was recorded after 1 hour of injection.

Group III: Test drug I (200mg/kg) was given orally for 14 days and on 14^{th} day after 30min of drug administration, Scopalamine (0.3mg/kg b.wt) was given i.p. TL was recorded after 1 hour of injection.

Group IV: Test drug II (400mg/kg b.wt) was given orally for 7 days and on 7^{th} day after 90min of drug administration, Scopalamine (0.3mg/kg b.wt) was given i.p. TL was recorded after 1 hour of injection.

PHYTOCHEMICAL ANALYSIS

The phytochemical analysis of various extracts were performed and presence offlavonoid, tannins, saponins and steroids were significant.

Table No 1: Qualitative Phytochemical analysis

SNO	TESTS	POWDER	HEXANE	ETHYL ACETATE	ETHANOL	AQUEOUS
1	Alkaloids	-	-	-	-	-
2	Flavonoids	+	+	+	++	+
3	Phytosterols	+	-	-	+	-
4	Triterpenoid	+	-	+	+	+
5	Tannins and phenolic compound	+	-	+	+	+
6	Saponins	+	-	-	+	+
7	Gums and mucilage	+	-	-	+	+
8	Carbohydrates	+	-	-	+	+
9	Glycosides	-	-	-	-	-
10	Proteins & amino acids	-	-	-	-	-
11	Steroids	+	-	+	+	+
12	Fixed oils and Fats	-	-	-	-	-

Note: + indicates presence, - indicates absence

PHARMACOLOGICAL STUDIES
IN VIVO ANTI ALZHEIMER ACTIVITY:

The Ethanolic extract of *Citrus maxima* (EECM) were selected based on the above *in vitro* studies. It was given on the Mice in the dose of 200 and 400 mg selected as 1/10 and 1/5 th of the dose which was proved to be non toxic in acute toxicity studies.

Mice were studied for transfer latency using Morris Water Maze and Y maze. The transfer latency are tabulated in table 22 & 23 and plotted in Fig 51& 52.

Table No 2: Effect of transfer latency using Morris Water Maze

SNO	GROUP	TREATMENT	ACQUISITION MEMORY (Sec)	RETENTION MEMORY Day 15 (Sec)
1	I	Negative control Scopalamine (0.3 mg/kg)	19.66 ± 1.632[b]	18.88±1.602 [a]
2	II	EECM 200 mg/kg	18.66 ± 1.96[a]	15.5 ± 1.51[b]
3	III	EECM 400 mg/kg	14.16 ± 1.60[a]	10.66 ± 0.80[a]
4	IV	Rivastigmine (0.3 mg/kg)	14.5 ± 1.37[a]	10.16 ± 1.47[a]

Mean ±SD, n=6.

All values are expressed as Mean ±SD and datas were analysed by One Way Annova followed by Dunnett's test a-P<0.05: b-P<0.01 when compared with controlgroup

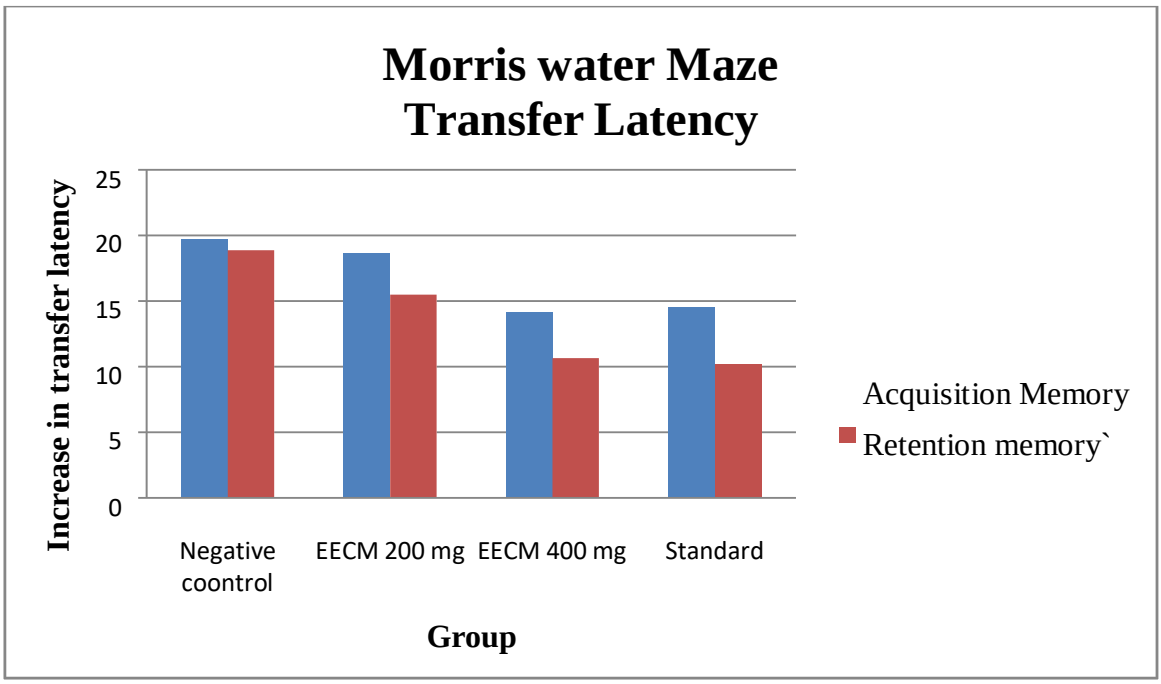

Fig 1: Morris water Maze Method.Y MAZE

Table No 3: Effect on Transfer Latency using Y Maze

GROUP	TREATMENT	% ALTERATION OF MICE
I	Negative control Scopalamine (0.3 mg/kg)	26.125± 0.63*
II	EECM 200 mg/kg	35.23 ± 0.75%**
III	EECM 400 mg/kg	38.54 ± 0.56%**
IV	Rivastigmine (0.3 mg/kg)	45.26 ± 0.43% *

% Alteration

Mean ±SD, n=6

%alternation= {(No. of alternations)x 100
(Total arm entries -2)}

All values are expressed as Mean ±SD and datas were analysed by One WayAnnova followed by Dunnett's test P*<0.05: P**<0.01 when compared with control group

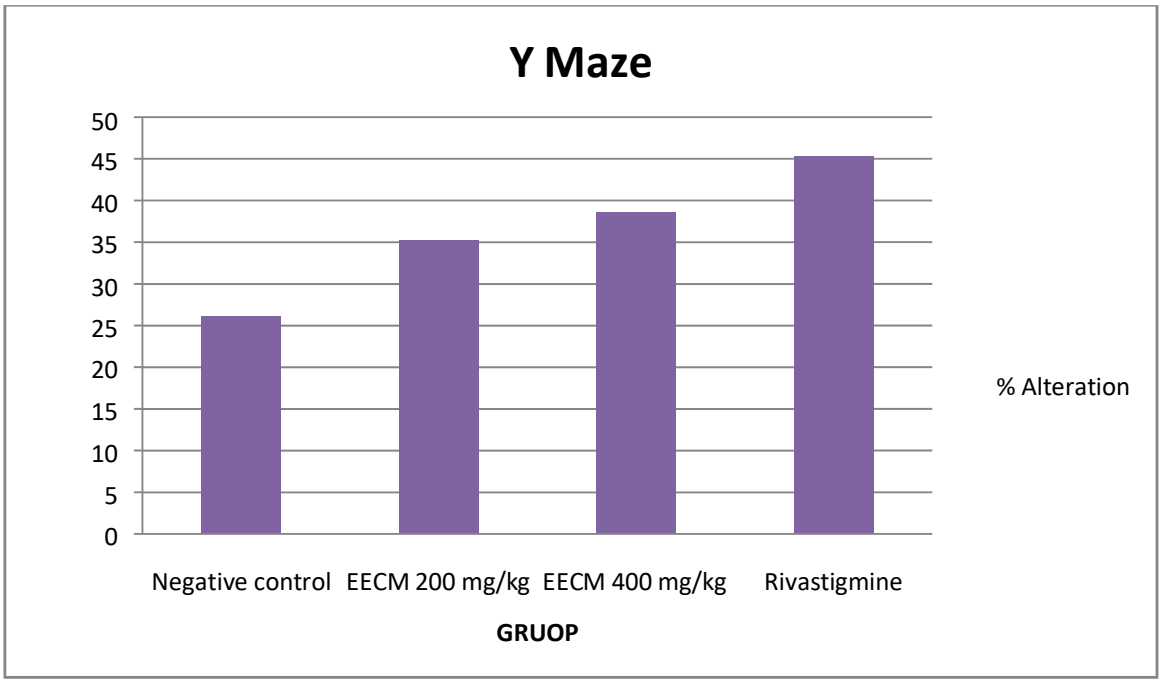

Fig 2: Effect on Transfer Latency using Y Maze

Study was done on Day 14 after the last dose of daily dose. Number of alterations and total arm entries were measured and Percentage alteration were calculated.

ESTIMATION OF BRAIN ACETYL CHOLINESTERASE ACTIVITY:

Brain is homogenized after the treatment and measured for the acetylcholinesterase level using Elman's method.

Table No 4: Brain Acetyl cholinesterase level

GROUP	TREATMENT	AChE Level Mmoles/Mg Protein
I	Negative control Scopalamine (0.3 mg/kg)	32.00±0.522[a]
II	EECM 200 mg/kg	19.00±0732[a]
III	EECM 400 mg/kg	22.00±0.593[b]
IV	Rivastigmine (0.3 mg/kg)	27.00±1.06[b]

Mean ±SD, n=6.

All values are expressed as Mean ±SD and datas were analysed by One Way Annova followed by Dunnett's test a-P<0.05: b-P<0.01 when compared with control group

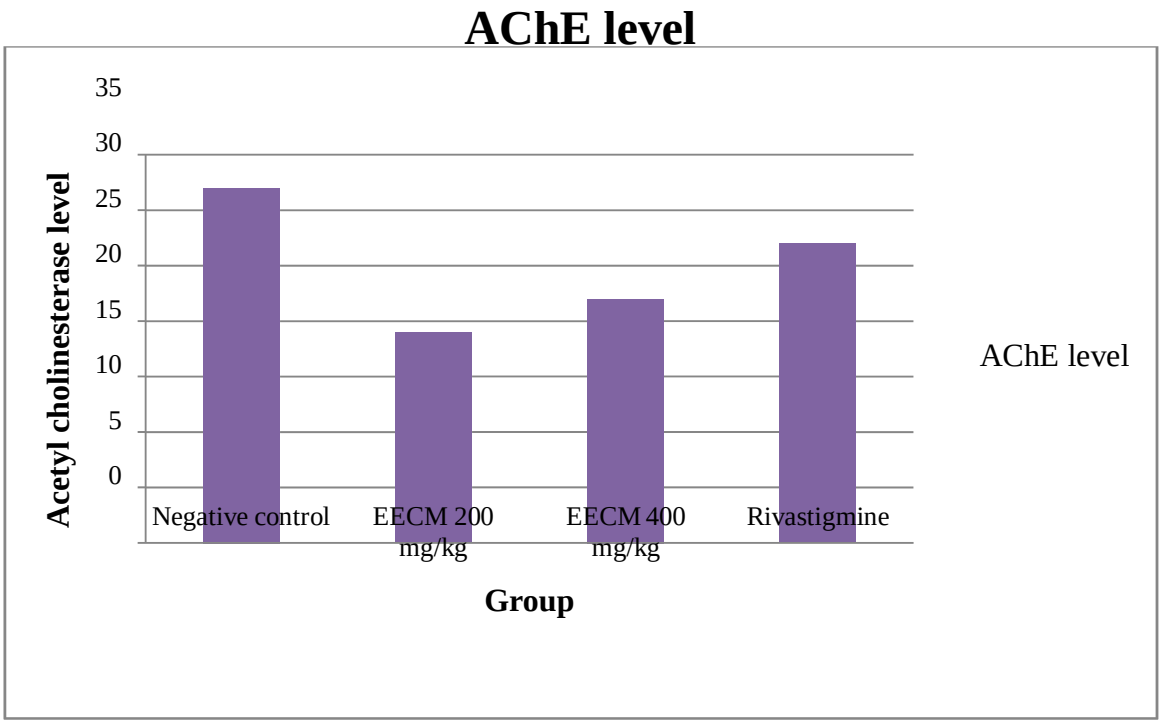

Fig 3: Brain acetyl cholinesterase level

HISTOPATHOLOGY:

Histopathology of the brain of various rats were analyzed and figured out.

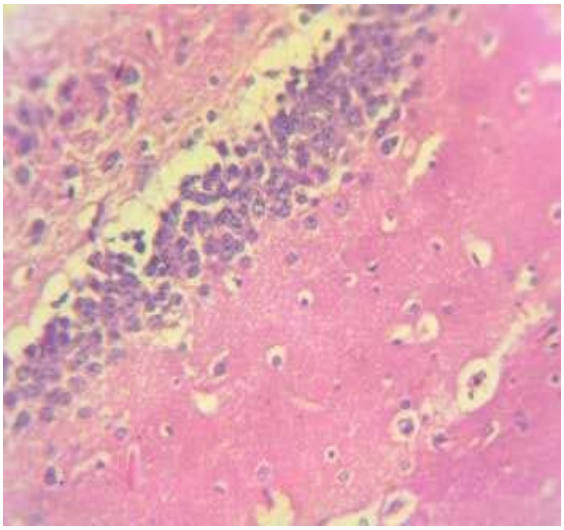

Fig 4: Histopathology of Group I (SCOPALAMINE INDUCED)

Slight increase in density of the neuronal cells

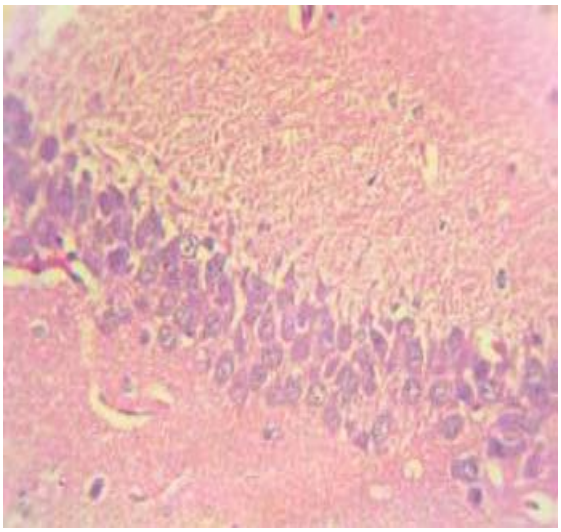

Fig 5: Histopathology of Group II (200mg/kg)

Significantly decrease in density of the neuronal cells

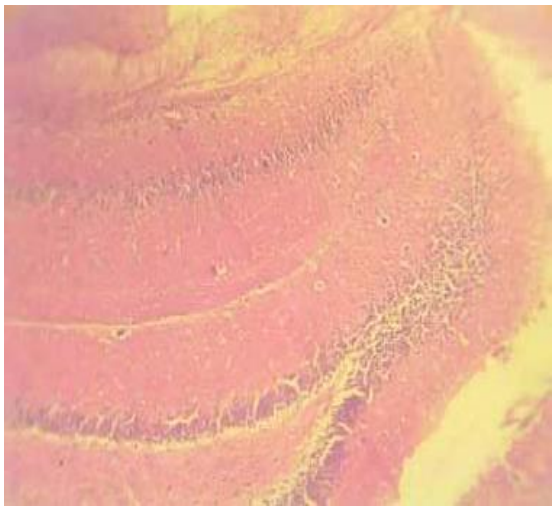

Fig 6: Histopathology of Group III

Significantly increase in density of the neuronal cell

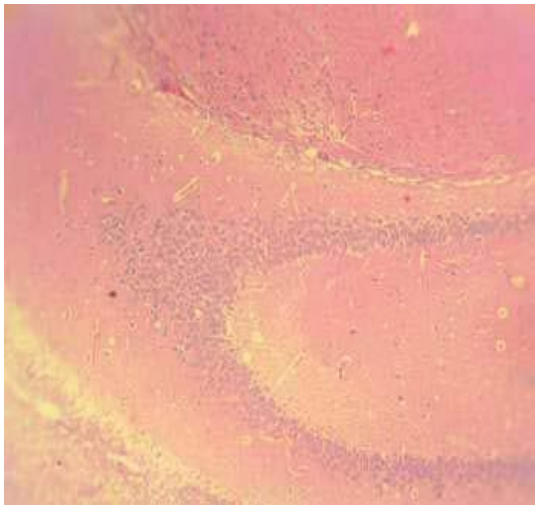

Fig 7: Histopathology of Group IV (400mg/kg) (Standard)

Normal density of neuronal cells.

SUMMARY

The literature survey showed that, only scrappy information was available on this shrub. With this scanty information on this plant consistent expectation of unexplored Phyto chemical profile and pharmacological efficacy under Rutaceae family forms the rationale for the study.

- Extraction and phytochemical evaluation of them deals with chemical analysis of the extract used for pharmacological screening. Qualitative preliminary phytochemical analysis was performed to detect the phytoconstituents nature and their presence in powder and its various extracts.
- Ethyl acetate extract showed the presence of **steroids, flavanoids, tannins, triterpenoid and phenolic compound.** Whereas the ethanolic extract showed the presence of **steroids, flavanoids, phenols, phytosterol, gums and mucilage, carbohydrate, triterpenoids, volatile oil.** Aqueous extract showed the presence of **steroids, flavanoids, phenols, gums and mucilage, carbohydrate and triterpenoid.**
- Based on the acute toxicity studies, previously reported 1/10th and 1/5th (200 and 400 mg/kg) of the maximum tolerated dose (2000 mg/kg B.W) were selected for the *in vivo* studies.
  The ethanolic extract of the peels of *Citrus maxima* was administered orally for fourteen days showed a **dose dependent and significant improvement in memory** of young mice and it also successfully reversed the memory deficits induced by Scopalamine. Furthermore a significant **decreased in cholinergic transmission level** in mice brain accounts for its multifarious beneficial effects such as anti alzheimer activity.
- Ethanolic extract showed the significant anti Alzheimer activity thus it was packed into a column to scrutinize the phytoconstituents present in it.

CONCLUSION:

Reports helps us to concluded that the pharmacognostical standards generated will be useful for the proper identification of plant and also to differentiate it from its closely related species and adulterants.

With the support of in vitro studies and Phytochemical screening, the ethanol extract was selected and subjected to in vivo studies.

The ethanolic extract with low and high dose in mice showed a significant activity anti Alzheimer activity. This may be due to one or more phytoconstituents like flavonoids present in it.

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