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Research Article

FORMULATION AND EVALUATION OF ORAL HERBAL TABLETS CONTAINING METHANOLIC EXTRACT OF *ALTERNANTHERA PUNGENS* WITH CYTOTOXIC ACTIVITY

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ABSTRACT

The purpose of the present study is to prepare methanolic extract of the whole plant Alternanthera pungens (MEAP), to perform phytochemical analysis, screening of cytotoxic activity using MCF-7 cell lines and to develop herbal tablets using various polymers. The methanolic extract was prepared by maceration method and by performing qualitative analysis of extract MEAP, it was found to contain alkaloids, steroidal and triterpenoids and flavonoids. The extract MEAP has exhibited significant cytotoxic activity by performing In-vitro studies by using MTT assay on MCF-7 cancer cells. The 9 herbal oral formulations were prepared using various polymers such as Eudragit L 100, HPMC E50 premium, HPMC K 100 LVCR. Among 9 prepared herbal formulations, the blend of all the herbal tablets have exhibited good flow properties such as angle of repose, bulk density, tapped density and also exhibited significant post compression parameters and evaluated for the quality control parameters as per I.P limits. FT-IR analysis of all 9 herbal tablets shown that there is no interaction between the MEAP and the polymers employed. For conclusion, the whole plant Alternanthera pungens is rich in flavonoids and triterpenoids and exhibited cytotoxic activity with the methanolic extract and their herbal formulations were exhibited significant values. Hence the presence of cytotoxic phytoconstituents, the extension of the isolation of bioactive compounds from the plant Alternanthera pungens may be the promising study to obtain lead molecules for future.

KEYWORDS: *Alternanthera pungens, herbal tablets, cytotoxic activity, Eudragit L 100, HPMC E50 premium, HPMC K 100 LVCR.*

INTRODUCTION

Medicinal plant drug discovery continues to provide new and important leads against various pharmacological targets including cancer, HIV/AIDS, Alzheimer's, malaria, and pain [1].

Pollution, unhealthy lifestyle, environmental toxins increases the risk of diseases. The side effects, overuse/misuses of allopathic drugs are also a major concern. World Health Organization, states that about 65% of the population across the globe prefer to use traditional and herbal medicines to treat disease [2]. The use of complementary alternative medicines has dramatically increased in India along with USA, in the last 2 decades [3]. Approximately 60% of anticancer agents are derived from medicinal plants and other natural resources however, there are still a number of plants that have an anticancer potential but they have not yet been fully investigated [4]. Of the 121 prescription drugs in use today for cancer treatment, 90 are derived from plants. Almost 74% of these, including taxol were discovered by investigating a folklore claim. Between 1981 and 2002, 48 out of 65 drugs approved for cancer treatment were natural products, based on natural products, or mimicked natural products in one form or another. These phytochemicals are commonly called chemotherapeutic or chemo preventive agents [5].

Cancer is a class of diseases characterized by out-of-control cell growth. There are over 100 different types of cancer, and each is classified by the type of cell that is initially affected. Cancer harms the body when altered cells divide uncontrollably to form lumps or masses of tissue called tumours. Cancer is a hyper proliferative disorder that involves transformation, dysregulation of apoptosis, proliferation, invasion, angiogenesis and metastasis. "Herbal formulation shall mean a dosage form consisting of one or more herbs or processed herb(s) in specified quantities to provide specific nutritional, cosmetic benefits, and/or other benefits meant for use to diagnose treat, mitigate diseases of human beings or animals and/or to alter the structure or physiology of human beings or animals".

Alternanthera pungens is a plant popularly called as Khaki weed. The leaves are widely used as vegetable. It is a kunth creeping, variable, prostate perennial pioneer plant of the Amaranthaceae L. family. This plant spreading by seed and vegetative, with roots often developing at the nodes of spreading stems, much-branched stems growing from a robust taproot, abundant in dry waste [6]. The plant can grow up to 50cm tall. The known hazard of this plant is suspected of causing the deaths of pigs and digestive disturbances and dermatitis of cattle. It is used as a bio-pesticide against stored grain pest [7]. The whole plant is to be used in gastric, hepatic and intestinal disturbances, such as dyspepsia, secretory and motor symptoms and the aerial part as diuretic and emollient. From the literature review, *Alternanthera pungens* was found to contain saponins, alkaloids, steroids, triterpenoids, leucoantho cyanidins, choline and exhibited spasmogenic properties, antimicrobial, diuretic, antidiarrhoeal, antidiabetic, antioxidant and anti-HIV properties [8]. The ethanolic extract of *Alternanthera pungens* whole plant contains C-glycoflavones which have proven their antioxidant properties and found to contain betalaines which have anticancer properties would promise to exhibit the beneficial anticancer activities for treating the cancer disease [8-10]. This study evaluated the cytotoxicity of methanolic extract of *Alternanthera pungens* and evaluation of herbal tablets with potent properties.

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MATERIALS AND METHODS

Plant Collection and Authentication: The Fresh whole plants of *Alternanthera pungens* was identified and collected in monsoon season by Prof. V.S. Raju, Plants systemic Laboratory, Department of Botany, Kakatiya University, Warangal, Telangana.

Preparation of methanolic extract of whole plant of *Alternanthera pungens:*

The crude powder (150gm) was macerated with methanol for 7days with occasional shaking. After 7days filtered and collected the extract. Again marc was remacerated for another 7 days. Final crude methanolic extract was collected, dried and weighed (42.6gm) and labelled as M and preserved in a dessicator for further drying.

Qualitative chemical analysis:

Sample preparation: 1 gm. of semisolid methanolic extract M was dissolved 100ml methanol and prepared a clear solution.

Table No. 1: Drugs, Cell lines and Chemicals

DMEM (Dulbecco's modified Eagles medium)	purchased from Sigma Chemicals Co. (St. Louis, MO)
MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]	purchased from Sigma Chemicals Co. (St. Louis, MO)
Trypsin	purchased from Sigma Chemicals Co.
EDTA Phosphate Buffered Saline (PBS)	purchased from Sigma Chemicals Co. (St. Louis, MO)
Fetal Bovine Serum (FBS)	Gibco.
25 cm^2 75 cm^2 flask and 96 well plated	purchased from Eppendorf India
MCF 7-Breast cancer cell lines	purchased from NCCS, Pune
HeLa and HepG2 cancer cell lines	purchased from NCCS, Pune
HPMC E50 & K100 PREMIUM LCVR	SD Fine Chemicals, Hyderabad
EUDRAGIT L100	SD Fine Chemicals, Hyderabad
PEG4000	SD Fine Chemicals, Hyderabad
SUCROSE	SD Fine Chemicals, Hyderabad
MG.STEARATE	SD Fine Chemicals, Hyderabad
MCC	Merk chemicals, Mumbai

Chemical tests:

1. Test for Alkaloids:

2ml of sample was defatted with 5% ethyl ether for 15 min. The defatted sample was extracted for 20 min with 5 ml of aqueous HCl on a boiling water bath. The resulting mixture was centrifuged for 10 min at 3000 rpm. 1 ml of the filtrate was treated with few drops of Mayer's reagent and a second 1 ml with Dragandroff's reagent and turbidity was observed.

2. Test for Saponins:

Froth test: 1ml was shaken with water in a test tube and it was warmed in a water bath and the persistent of froth indicates the presence of saponins.

3. Test for phenols:

Ferric chloride test: 1ml sample was stirred with 10 ml of distilled water. This was filtered and ferric chloride reagent was added to the filtrate, a blue, green, black precipitate formation is indicated for the presence of phenols or enols.

4. Test for flavonoids:

Schinoda test: To 1ml of sample add magnesium turnings, 1ml of dil. HCl then pink color production is indicated for the presence of flavonoids and their glycosides.

5. Test for Terpenoids and Steroids:

Liebermann-butchard test: 2 ml of the sample was treated with 0.5 ml of acetic acid anhydride and cooled in ice. This was mixed with 0.5 ml of chloroform and 1 ml of concentrated sulphuric acid was then added carefully by means of a pipette. At the separations level of the two liquids, a reddish-brown ring formation is indication for the presence of triterpenoids or steroids.

6. Test for carbohydrates and Glycosides:

Molish test: 10 ml of 50% HCl was added to 2 ml of methanolic extract in a test tube. The mixture was heated in a boiling water bath for 30 min. 5 ml of Fehling's solution was added and the mixture was boiled for 5 min to observe a brick red precipitate which is an indication for the presence of carbohydrates.

7. Test for Proteins:

Biuret test: To 1ml of sample add 1ml of 40% NaoH and 1ml of 1% Cuso4 solution, shake well. Violet color produced at the junction of 2 layers indicates the presence of proteins.

Xanthoproteic test: To 1ml of sample add 0.5ml of conc.HNO3, boil it, yellow precipitate was produced, then add ammonia, yellow precipitate turned into orange colour indicates the presence of protein.

Thin Layer Chromatography (TLC):

TLC studies were performed for the detection of Phytochemicals such as Alkaloids, Terpenoids, Steroids and flavonoids by the following procedures:

Sample preparation: 0.5gm of methanolic extract (M) in 100ml of methanol.

1. For Alkaloids:

Mobile phase: Toulene: Ethyl acetate: diethylamine (70:30:10)

Detection: 1. bluish color fluorescence at UV 254nm; 2. Spray with Dragondorff's reagent. (Orange /red spots)(Plate 1)

2. For Triterpenoids and flavonoids:

Mobile phase: Methanol: water (90:10)

Detection: 1. yellow, blue or green florescence at UV 365nm. 2. Heat at 100˚C followed by spraying with Vanillin sulphuric acid (VS) reagent. (Plate-2)

3. For steroidal compounds and its glycosides:

Mobile phase: Ethyl acetate: Methanol: Water (100:13.5:10)

Detection: blue spots on heating followed by LB reagent. (Plate-3)

MTT assay of MEAP:

MTT Assay is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, dark purple coloured formazan crystals. The cells are then solubilized with a DMSO and the released, solubilized formazan reagent is measured spectrophotometrically at 570 nm.

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$$\%\text{Inhibition} = \frac{100(\text{Control}-\text{Treatment})}{\text{Control}}$$

Procedure:

Cell viability was evaluated by the MTT Assay with three independent experiments with six concentrations of compounds in triplicates. MCF 7 A cells were trypsinized and perform the tryphan blue assay to know viable cells in cell suspension. Cells were counted by haemocytometer and seeded at density of 5.0×10^3 cells/well in µl media in 96 good well plate culture medium and incubated overnight at 37^0C. After incubation, remove the old media and add fresh media 100 µl with different concentrations of test compound in representative wells in 96plate.After 48hours, discard the drug solution and add the fresh media with MTT solution (0.5 mg/mL^{-1}) was added to each well and plates were incubated at 37^0C for 3 hours. At the end of incubation time, precipitates are formed as a result of the reduction of the MTT salt to chromophore formazan crystals by the cells metabolically active mitochondria. The optical density of solubilized crystals in DMSO was measured at 570 nm on a microplate reader. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% values is generated from the dose response curves for each cell line.

Determination OF UV Absorption maxima: MEAP solution was prepared in 0.1 N HCL and diluted suitably. The UV spectrum of the solution was taken on Lab India 3200 UV/Vis double beam Spectrophotometer. The Solution exhibited UV maxima at 243 nm. The procedure was repeated with pH 6.8 Potassium dihydrogen phosphate buffer.

Formulation of MEAP Tablet by Direct- Compression: Composition of preliminary trials for MEAP Conventional Tablets by direct compression is shown in table 2. All the ingredients were weighed. The blend is compressed using rotary tablet machine-8 station with 12mm flat punch.

Table No. 2: Formulation of MEAP of herbal tablets

INGREDIENT	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9
MEAP	60	60	60	60	60	60	60	60	60
HPMCE50PREMIUM	40	80	120	-	-	-	-	-	-
HPMC K100 LVCR	-	-	-	40	80	120	-	-	-
EUDRAGIT L 100	-	-	-	-	-	-	40	80	120
SUCROSE	20	40	60	20	40	60	20	40	60
MG.STEARATE	5	5	5	5	5	5	5	5	5
MCC	175	115	55	175	115	55	175	115	55
TOTAL	300	300	300	300	300	300	300	300	300

All ingredients are expressed in mg only for a single tablet.

RESULTS AND DISCUSSION

Percentage Yield of MEAP:

Table No. 3: Percentage Yield of MEAP

S.No.	Sample Type	Physical status	Percentage Yield (w/w)
1.	MEAP ----- Methanolic Extract	Green colour semi solid	28.4%

Qualitative analysis of the phytochemicals of *Alternanthera pungens* (MEAP):
1) From Chemical tests:

Table No. 4: Phyto Constituents present in the Alternanthera pungens

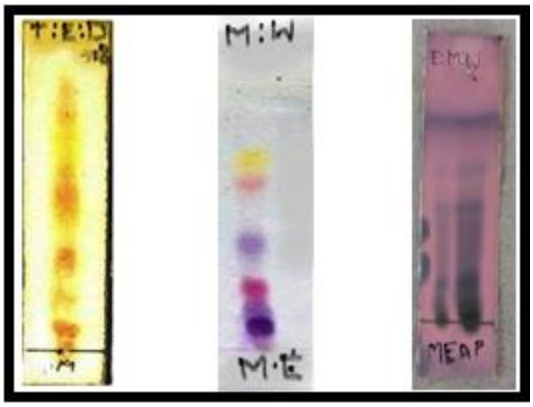
S.No	Phyto constituents	*Alternanthera pungens (MEAP)*
1.	Flavonoids	+
2.	Alkaloids	+
3	Saponins	-
4	Phenolic compounds or Tannins	-
5	Terpenoids	+
6	Steroids	+
7	Glycosides	+
8	Anthraquinone	-
9	Protein	-
10	Carbohydrate	+

Table No. 5: Detection of Phytoconstituents by TLC

S. No.	Name of Test	Observation	Inference
1.	**PLATE-1** **a. Dragondorffs test** **b. TLC** ***Mobilephase:*** Toulene:Ethyl acetate: diethylamine (70:30:10)	a. Reddish brown color was produced. **b. Detection by TLC:** **Rf values:** 0.01,0.12,0.15,0.2,0.35,0.37, 0.47,0.5,0.51,0.85,0.9	**a.** indicates the presence of alkaloids. b. confirms the presence of alkaloids Indicates the presence of flavonoids.

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	PLATE-2 **1. Schinoda test**	1. bluish color fluorescence observed at UV 254nm 2. Orange to red spots were observed on Spray with Dragondorff's reagent. Positive- pink color produced in solution.	a. Indicates the presence of triterpenoids or steroids.
2.	**2.Liebermann-Burt chard test** **b. TLC plate** ***Solvent system:*** Methanol:water (90:10) ***Detection:*** VS **reagent**	a. Positive - blue color solution was observed. **b. Rf value -** 0.2, 0.3, 0.37, 0.51, 0.75, 0.8 (no. of spots of pink to red colour were seen on spraying the plate with V-S reagent and on heating turned to yellow)	b. Blue, red to pink color spots indicates the presence of triterpenoids Yellow spots show the presence of flavonoids.
3.	**PLATE-3** **a. LB test** **b. TLC** ***Mobile phase:*** E:M:W (81:11:7)	a. Positive - blue color solution was observed ***Detection:*** Blue spots on heating followed by LB reagent	a. Indicates the presence of steroidal compounds. b. Presence of steroidal and glucosides.



TLC Plate 1 **TLC Plate 2** **TLC Plate 3**

Fig. 1: Detection of Phytoconstituents by TLC

Pharmacological Evaluation of Methanolic Extract of *Alternanthera Pungens:*
Screening for anticancer bioactivity by In-vitro studies:

Table No. 6: MTT assay of MEAP

S. No.	Compound in MCF 7	IC_{50} (µg/ml)
1	MEAP	10.28

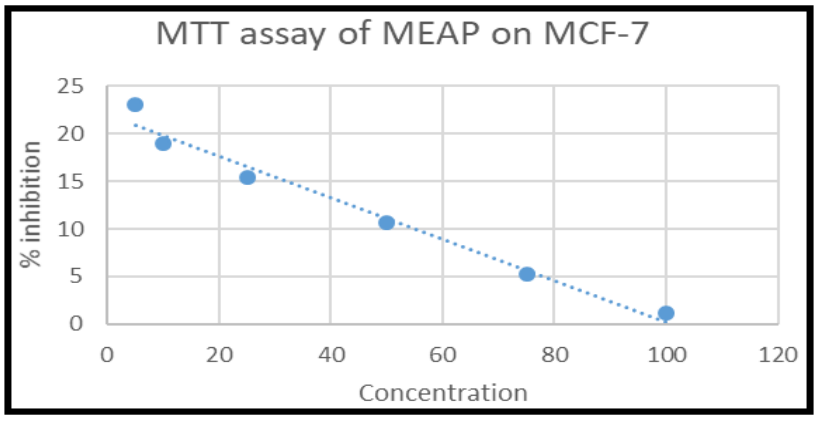


Fig. 2: Cytotoxic effect of the sample MEAP on MCF7 Cell line
MTT assay of extract MEAP exhibited the significant inhibitory effect on MCF-7 cancer cells

Herbal Tablets:
Standard Calibration curve of MEAP:

It was found that the estimation of MEAP by UV spectrophotometric method at λ_{max} 243 nm in 0.1N Hydrochloric acid had good reproducibility and this method was used in the study. The correlation coefficient for the standard curve was found to be closer to 1, at the concentration range 5-25µg/ml.

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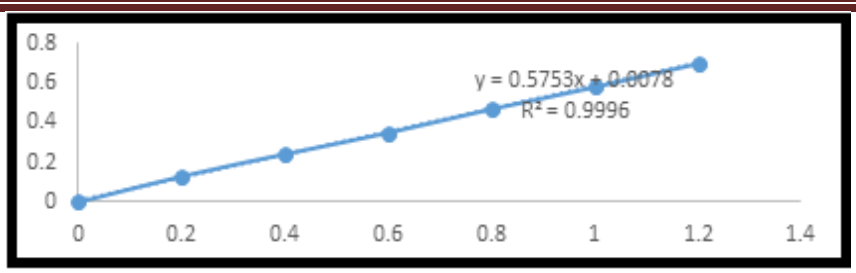


Fig. 3: Standard graph of MEAP in 0.1 N HCl

It was found that the estimation of MEAP by UV spectrophotometric method at λ_{max} 265 nm in pH 6.8 Phosphate buffer had good reproducibility and this method was used in the study. The correlation coefficient for the standard curve was found to be closer to 1 at the concentration range 2-12µg/ml.

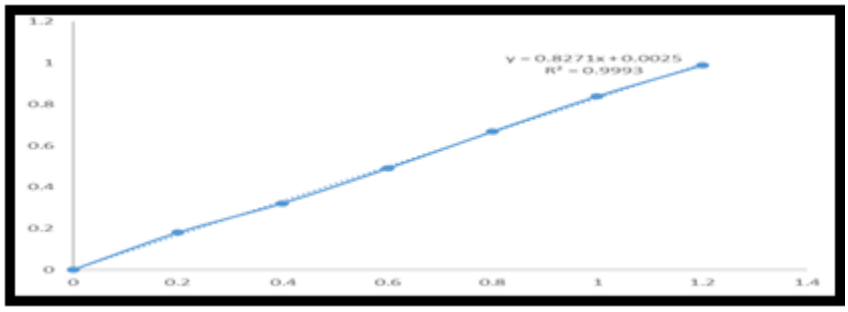


Fig. 4: Standard graph of MEAP in pH 6.8 Phosphate buffer

Evaluation Parameters for Extended release tablets of MEAP:

The values for angle of repose were found in the range of 23.14°-23.65°. Bulk densities and tapped densities of various formulations were found to be in the range of 0.82 to 0.86 (gm/cc) and 0.54 to 0.59 (gm/cc) respectively. Carr's index of the prepared blends fall in the range of 16.24% to 16.95%. The Hausner ratio n fall in range of 1.32 to 1.37. From the result it was concluded that the powder blends had good flow properties and these can be used for tablet manufacture.

Post compression Parameters:

Weight variation test: Tablets of each batch were subjected to weight variation test, difference in weight and percent deviation was calculated for each tablet. The average weight of the tablet is approximately in range of 298 to 305 mg, so the permissible limit is ±5% (>220 mg). The results of the test showed that, the tablet weights were within the pharmacopoeia limit.

Hardness test: Hardness of the three tablets of each batch was checked by using Pfizer hardness tester and the data's were shown in Table 6.8. The results showed that the hardness of the tablets is in range of 2.8 to 2.9 kg/cm^2, which was within IP limits.

Thickness: Thickness of three tablets of each batch was checked by using Vernier Caliper. The result showed that thickness of the tablet is raging from 4.5 to 4.6 mm.

Friability: The average friability of all the formulations lies in the range of 0.34 to 0.56% which was less than 1% as per official requirement of IP indicating a good mechanical resistance of tablets.

In-vitro Dissolution studies: The Oral herbal tablets subjected to In-vitro dissolution studies using USP dissolution apparatus at 37°C, 50 rpm speed, 900 ml of 0.1N Hcl and water was used as dissolution medium. Dissolution was withdrawn keeping the tablet in the dissolution basket by using paddle method for 2 hours. Then pH 6.8 phosphate buffer was added to the dissolution medium (900ml) and the dissolution was carried out for about 6 hours.The samples were withdrawn at regular time intervals of 30 min, 1 hour, 2 hr, 3, 4, 5, 6, 7 & 8 hours respectively.

From the above results it was evident that the formulation F8 is best formulation with desired drug release pattern extended up to 8 hours.

Application of Release Rate Kinetics to Dissolution Data:

Various models were tested for explaining the kinetics of drug release. To analyze the mechanism of the drug release rate kinetics of the dosage form, the obtained data were fitted into zero-order, first order, Higuchi, and Korsmeyer-Peppas release model.

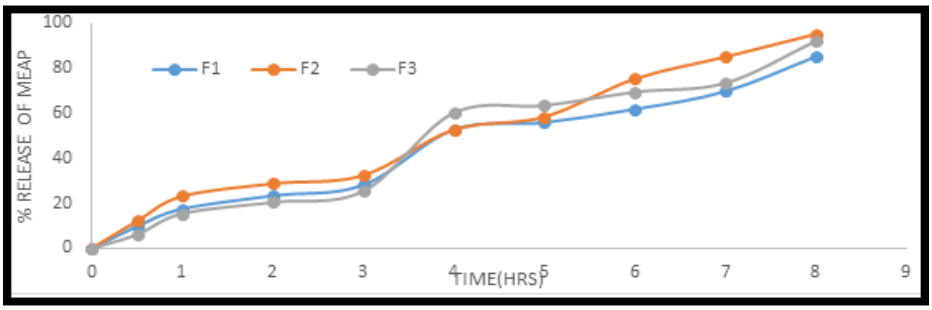


Fig. 5: Dissolution profile of formulations prepared with HPMC E50 PREMIUM polymer

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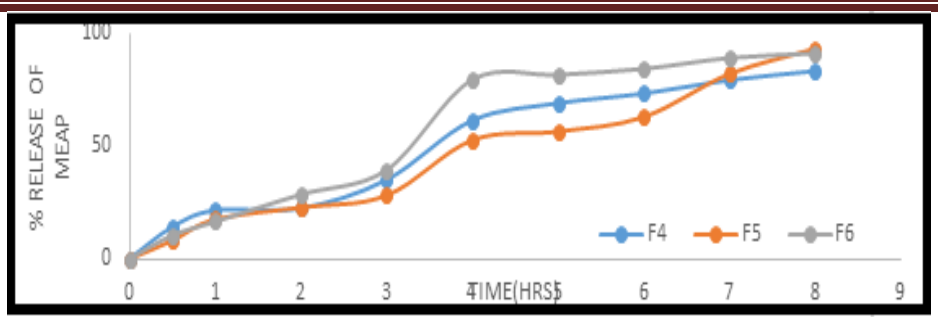


Fig. 6: Dissolution profile of formulations prepared with HPMC K100 LCVR PREMIUM

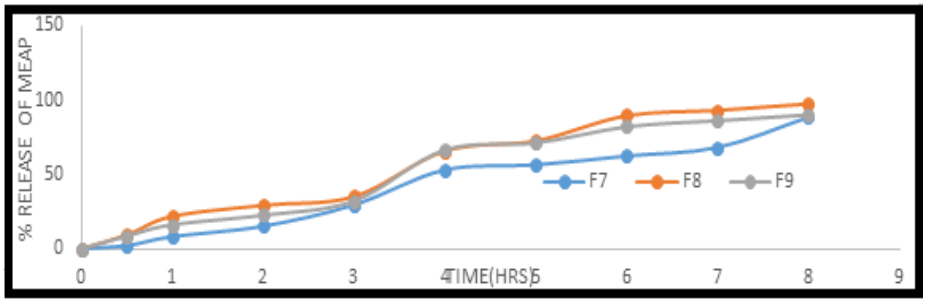


Fig. 7: Dissolution profile of formulations prepared with EUDRAGIT L100 as polymer

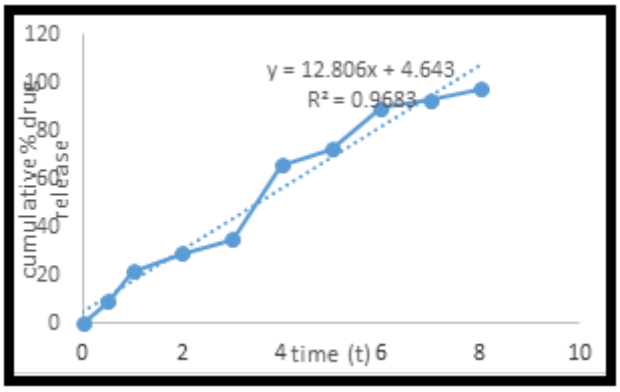


Fig. 8: Zero order release kinetics

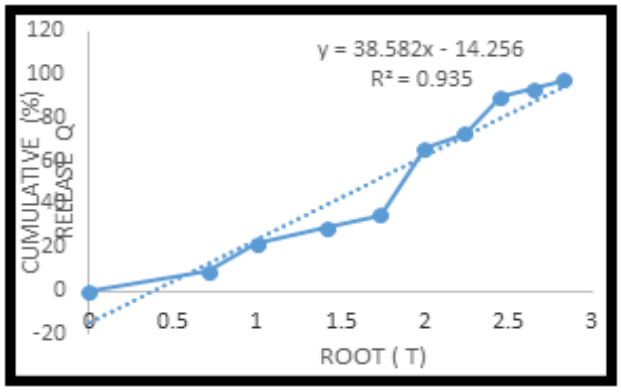


Fig. 9: Higuchi order release kinetics

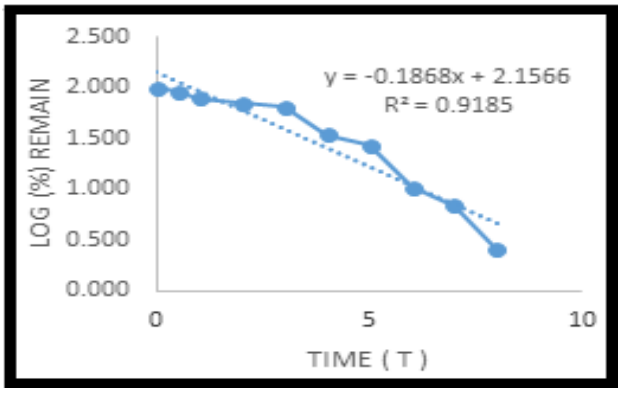


Fig. 10: Peppas order of release kinetics

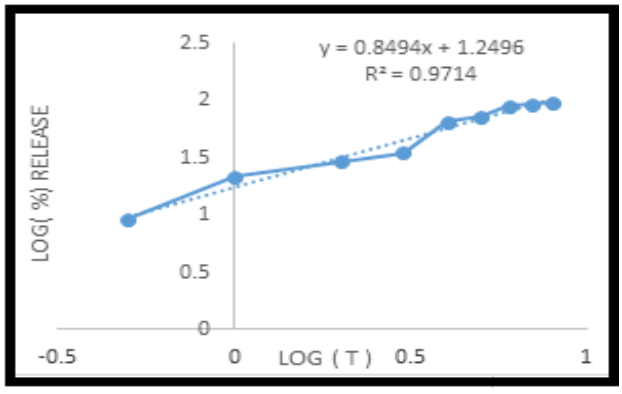
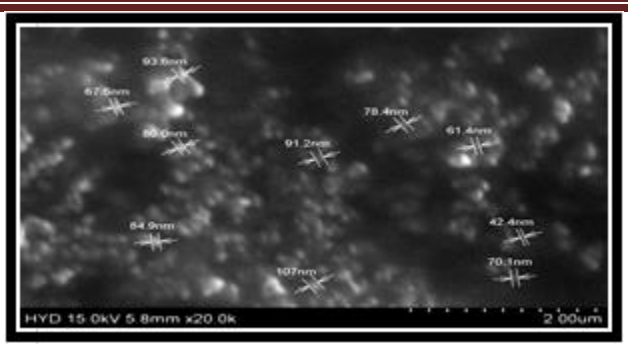


Fig. 11: First order release kinetics

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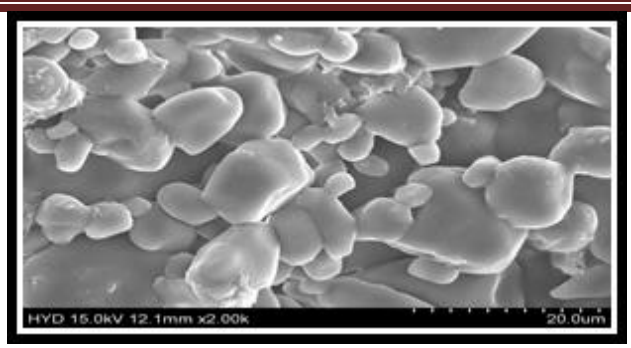


Fig. 12: SEM Analysis

These Images are given information about the surface topography of Oral herbal tablet and Composition of sample MEAP where the presence of mixture of compounds with different size ranges varies from 42.4nm to 107nm.

Compatability studies by FTIR:

The IR spectrum of MEAP exhibited characteristic band at 1739 cm^{-1} for carbonyl group, 1020-1043 cm^{-1} for O-R stretching and a broad band at 3446 cm^{-1} for hydroxyl group. By observing the above FTIR graphs of 9 herbal formulations of MEAP with various polymers there is no considerable diffraction of the characteristic peaks produced by MEAP. It is concluded that there is no interaction of polymers with extract MEAP when they employed and formulated.

CONCLUSIONS

From this study, it is concluded that prepared herbal tablets were exhibited cytotoxic activity with desired drug release and extended up to 8hours. For conclusion, the whole plant *Alternanthera pungens* is rich in flavonoids and triterpenoids with cytotoxic properties would promising work to isolate the lead compounds for future use.

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