RESEARCH PAPER

Screening for Phytochemicals having Herbicidal Properties from Pomegranate Peel

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ABSTRACT
One of the chief threats to current agricultural sector is herbicide resistant plants resulting from the usage of synthetic herbicides. The other drawbacks of these synthetic herbicides comprise their expensive cost and environmental damage which destroys the essential soil biota. In order to address these limitations, the current work is aimed at developing a sustainable, eco-friendly and cost-effective herbicide. Pomegranate (*Punica granatum*) peel is having high phenolic content and has been investigated for its allelopathic, antibacterial and antioxidant benefits. To explore the herbicidal activity of pomegranate peel, germination studies were conducted on prominent weeds such as *Tridax procumbens, Galinsoga parviflora*, *Spermacoce ocymoides and Parthenium hysterophorus.* The extract exhibited inhibitory effects specific to the concentration where higher concentration at 30 mg/ml led to suppression of germination and lower concentration at 5 mg/ml led to growth promotion in particular species. Uptake kinetics were calculated using total phenol quantification implying a significant interaction between the plant tissues and peel extract. Biochemical and physiological characteristics such as chlorophyll content and lipid peroxidation were evaluated where elevated malondialdehyde represented the oxidative stress and reduced chlophyll content showed the photosynthetic damage. Scanning electron microscopy (SEM) studies showed the morphological variations in leaf structure especially stomatal alterations. The formulation analysis exhibited beneficial herbicidal properties specifically acidic pH, fast wettability and suspensibility of 99.86% which contribute towards an effective application. An increased dehydration of *Parthenium hysterophorus* weed post treatment was observed during field trials indicated the herbicidal activity. In conclusion, the outcomes support the extract of pomegranate peel as a viable, harmless alterative for synthetic herbicides with enormous potential for sustainable agriculture applications.

Keywords: Pomegranate peel, Herbicide, Seed germination, SEM, Antioxidant
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INTRODUCTION

Pomegranate is known to be a miracle fruit since time immemorial. Some of its supreme properties include its antioxidant and anti-microbial property. The anti-microbial and antioxidant property of pomegranate peel was established during the current work. Qualitative tests were performed for identification of major phytochemicals present in the pomegranate peel extract. Total Phenolic Content was estimated using Folin-Ciocalteau method with Gallic acid was the standard. Anti-Microbial activity (with bacteria and fungi) was carried out on two bacterial strains (*Escherichia coli* and *Staphylococcus aureus*) and two fungal strains (*Aspergillus niger* and *Candida bombicola*). Anti-Oxidant activity of pomegranate peel was established by utilising iron reducing power assay while the and free radical scavenging property of pomegranate peel was determined by measuring hydrogen peroxide radical scavenging activity.

Pomegranate peel also has a great potential to be used as an herbicide as well as a fungicide due to the presence of a high concentration of phenolic compounds. Allelopathy is one such phenomenon that enhances the herbicidal properties of pomegranate peel. It is a biological phenomenon by which a plant or an organism produces one or more bio-chemicals that influence the germination, growth, survival, and reproduction of other plants or organisms. Research has shown that allelochemicals from ethyl acetate extract of pomegranate peel have inhibitory effect on growth of algal species. [1] The non-toxic and environment friendly property of pomegranate peel helps to overcome the limitation of synthetic herbicides that contaminate the soil and cause a disruption of natural biota. [2]

Using these properties of pomegranate peel extract as a basis for the proposed work, we intended to formulate an organic, eco friendly herbicide by using techniques such as mass spectrometry for analysis of phenolic compounds present in the pomegranate peel extract, performing germination studies to study the effect of pomegranate peel on the growth of seeds, quantifying the effect of phenols present in the extract on the phenols present in the chosen weeds, carrying out studies on soil biota to understand the impact of our herbicide on the natural ecosystem as well as carrying out studies on extent of lipid peroxidation and chlorophyll content to monitor the effect of the herbicide on

the chosen weeds.

According to a protocol, TCA/Acetone precipitation method was used to purify proteins from cell lysates containing some contaminants (salts, nucleic acids, etc.). The protocol was based on using ice-cold acetone for precipitating out the proteins. Trichloroacetic acid (TCA) used in this method is mainly for partial unfolding of proteins and to dehydrate the hydration shell around the proteins. This makes precipitation of the proteins easier. Addition of the sample, acetone and TCA was in the ratio 1:8:1 (ml) respectively. After centrifugation at 11500 rpm, the pellet contained the proteins and the supernatant contained contaminants. Further steps were performed since the final product of interest was protein in the respective method. [3]

Identification and analysis of compounds present in a target extract helps to identify the active ingredient present in an herbicide. Mass spectrometry is one of the most efficient techniques used for this analysis. A study carried out that highlights the importance of mass spectrometric analysis, involved, the identification of bioactive compounds present in ethyl acetate extract of pomegranate rind. Column chromatography was carried out with solvents of different polarity in order to obtain elute with a high concentration of compounds. The ethyl acetate extract was then used for GS-MS (Gas Chromatography- Mass Spectrometry) analysis. A SHIMADZU QP2010 system equipped with Elite-1 fused silica capillary column (Length: 30m, Diameter: 0.25mm, Film thickness: 0. 25 µm composed of 100% Dimethyl poly siloxane) was used with an ionization energy of 70eV. Helium gas (99.99%) was used as the carrier gas. The database of National Institute Standard and Technique (NIST08s), WILEY8 and FAME were used to interpret the results obtained. The analysis yielded Pyrogallol (41.88%), 5-Hydroxymethylfurfural (14.10%), D-Allose (9.17%), 2-Methoxy-1, 4-Benzenediol (8.34%) and 2, 3 Dimethylfumaric acid (3.96%) as the main bioactive compounds present in pomegranate rid. [4]

Studies on germination with ANOVA analysis were performed. The assay is carried out as follows - Fifty seeds were germinated in petriplates on Whatman filter paper with 5ml of the aqueous extract (sample) or distilled water as control. Three replicates were incubated in a randomized complete block design at 20°C in an incubator with fluorescent light. Germination percentages were recorded and total seedling length was measured after 5 days of incubation (using 5 seeds chosen at random). [5]

The percentage germination inhibition was calculated based on a simple formula which involves shoot length of the control and test sample.

Inhibition of seed germination, (%) = [(C-T) / C] x 100

where,

C: represents number of weed seed germination on the control plate.

T: represents number of weed seed germination on the treated plate. [2]

According to a study, Scanning Electron Microscopy (SEM) was performed to observe structural changes in chloroplasts of Broccoli during senescence. The sample preparation was carried out using freeze fracture technique. To understand better about chloroplast degradation mainly during senescence Transmission Electron Microscopy (TEM) was also performed along with SEM. SEM showed that stroma thylakoids in the chloroplast initially were parallel to each other and grana thylakoids were tightly stacked. As senescence advanced, the grana thylakoids degenerated and formed globules which in turn formed vesicles that were expelled into cytosol. [6]

One of the most important characteristic features of an efficient herbicide is its ability to be taken up by the weed. Uptake kinetics plays a major role in the mechanism of action of an herbicide. Pomegranate peel extract has a high concentration of phenolic compounds and hence the extent of uptake of the extract by the weeds can be monitored by quantifying the amount of phenolic compounds in the weed before and after application of the extract on the weeds as an herbicide. One of the studies carried out in order to establish the efficacy of pomegranate peel as an herbicide quantified total phenol content using the Folin-Ciocalteau method of determination of total phenols. One mg of pomegranate crude extract was dissolved in 10 ml of 80% methanol and the methanol was allowed to evaporate by agitating the mixture for 15 min over a hot plat at 50˚C. The volume was then adjusted to 10 ml using distilled water. 250 µl of Folin-Ciocalteau reagent (1N) was added to each solution and kept at room temperature after which the absorbance of each reaction mixture was measured using a spectrophotometer at 725 nm. Gallic acid was used as the standard. [7]

Pure herbicide molecules have a limited end user application. In order to enhance their activity and efficiency, they are often combined with surfactants or other solvents in order to form a suitable formulation. The perfect formulation of an herbicide not only enhances its phytotoxicity but can also enhance its shelf life, its persistence in soil and resistance to the harsh environment. [8]

One such study was carried out to in order to determine the ability of pomegranate peel extract and henna extract to kill certain weeds. Dried crude extracts of acetone and methanol were dissolved in acetone and added to a powder made of kaolinite: diatoms earth: surfactant (45:45:10) with a ratio of (3:7), then slowly mixed by adding more acetone until it was completely mixed. The surfactant used was Tween 80: Tween 20 in the ratio of 1:1. A good suspensibility, wettability and pH of 61.0 and 4.0 and 6.41 were obtained for acetone formulation of henna and 65.0, 3.0 and 6.9 were obtained for the methanol formulation of henna, respectively. The corresponding values for pomegranate extract were 57, 3.0 and 6.77 for the acetone formulation and 62.0, 4.0 and 6.71 for the methanol formulation, respectively. [1]

Phenolic compounds have superior antioxidant properties. This property of phenolic compounds leads to excessive lipid peroxidation and eventually the damage of tissues. The levels of lipid peroxidation in older plants infected by virus and those which are subjected to heat stress were studied in

order to establish the relationship between lipid peroxidation, viral disease and abiotic factors . The level of lipid peroxidation in the leaf tissue was measured in terms of malondialdehyde (a product of lipid peroxidation) content determined by the thiobarbituric acid (TBA) reaction. It was shown that a viral infection significantly reduces the content of TBA-active products in older plants and the content of malonadialdehyde significantly increased under conditions of heat stress in plants with symptoms of viral infection. [9]

Chlorophyll content plays a major role in the growth of a plant. Thus, it forms a beneficial parameter to monitor the growth of a plant. A good herbicide must cause gradual degradation in the chlorophyll content of a plant, causing a reduction in the rate of photosynthesis and thus eventually leading to the death of the plant. One of the protocols modified to efficiently quantify chlorophyll content includes the use of 300mg of leaves which are cut into small pieces and ground after the addition of liquid nitrogen. 5ml of 80% acetone was then added to a 15ml falcon tube and the powder was transferred into it. The mixture was then mixed in the dark for 15-30 minutes. This step was followed by centrifugation at 4˚C for 15 minutes at 3000rpm and the supernatant was transferred to a new centrifuge tube and kept in the dark. The supernatants were then mixed and the absorbance of the mixture was measured using a spectrophotometer. The concentration of chlorophyll in the leaves was then calculated as follows:

Chlorophyll a (mg/g) = $(12.7 \times A_{663} - 2.69 \times A_{645}) \times V/1000 \times W$

Chlorophyll b (mg/g) = $(22.9 \times A_{645} - 4.86 \times A_{663}) V/1000 \times W$

Chlorophyll a+b (mg/g) = $(8.02 \times A_{663} + 20.20 \times A_{645}) \times V/1000 \times W$

Where,

A= Absorbance, V=Volume of extract (ml), W=Weight of leaves (g). [10]

1. METHODOLOGY

1.1 Purification of phytochemicals extracted from pomegranate peel.

The extract, 100% ice-cold acetone and 100% trichloroacetic acid (100% TCA, w/v) respectively were mixed in the ratio 1:8:1 i.e. 1ml of the extract, 8ml of 100% ice-cold acetone and 1ml of 100% TCA. This was precipitated at -20°C for 1 hour and then centrifuged at 11,500 rpm (18000 x g) for 15min at 4°C in a microcentrifuge. The pellet was discarded and the supernatant which is the purified form of the crude extract was collected and stored for further use.

1.2 Germination Studies

Fifty seeds are germinated in petriplates on whatman filter paper with 5ml of the aqueous extract (sample) or distilled water as control. Three replicates are incubated in a randomized complete block design at 20°C in an incubator with fluorescent light. Germination percentages are recorded and total seedling length is measured after 5 days of incubation (using 5 seeds chosen at random).

The percentage germination inhibition was calculated based on a simple formula which involves shoot length of the control and test sample.

Inhibition of seed germination, (%) = [(C-T) / C] x 100

where,

C: represents number of weed seed germination on the control plate.

T: represents number of weed seed germination on the treated plate.

1.3 Scanning Electron Microscopy (SEM) Analysis.

The leaves of the five weeds and chilli plant were dried. The dried leaves were mounted on carbon tape which was stuck on pin mount specimen holders. Gold Sputtering (Ultra thin coating of an electrically conducting material) was done to make the sample conductive in nature using a sputter coater instrument. The process was carried out for 75 seconds. After the sputtering process, the samples were placed inside the chamber of the main SEM unit and photographs were taken for observations.

1.4 Study of uptake kinetics using Total Phenolic Content Quantification:

Increasing concentrations of gallic acid were taken in test tubes and the volume was made up to 1 ml with distilled water. 0.5ml of the FC reagent was then added to each test tube followed by the addition of 2.5ml of 20% sodium carbonate. The test tubes were then vortexed and incubated in the dark for 40 minutes. After incubation, the absorbance of each of the reaction mixture was measured at 725 nm. Simultaneously, the procedure was repeated with 1 ml of stock solution of chilli plant leaf macerated in water.

1.5 Characterisation of extracted herbicide.

Test for suspensibility:

0.625 g of dried pomegranate peel extract was added to 24.25 ml of hard water in a measuring cylinder and the volume was adjusted to 25 ml. The cylinder was then covered and inverted vertically 30 times for one minute. It was left undisturbed for one hour following which 2.5 ml of the solution at bottom was collected and dried in an oven at 100˚C and the residue was then weighed.

Test for pH variation:

0.25 g of dried pomegranate peel extract was taken in a 50 ml eppendrof tube containing 12.5 ml deionised water and vortexed at high speed until the powder completely dissolved. The volume was then made up to 25 ml, and the pH was measured using a pH meter.

Test for wettability:

0.25 g of powder was dropped in 12.5 ml of distilled water placed in a Petri dish until the powder was completely wet. Time taken for the powder to become completely wet was recorded.

1.6 Lipid Peroxidation.

0.25 g leaf sample was homogenized in 5 ml 0.1% TCA. The homogenate was centrifuged at 10,000 g for 5 min. To

1ml aliquot of the supernatant 4 ml 20% TCA containing 0.5% TBA was added. The mixture was heated at 95 °C for 30 minutes and then quickly cooled in an ice-bath. After centrifuging at 10,000 g for 10 minutes, the absorbance of the supernatant at 532 nm was read and the value for the non-specific absorption at 600 nm was subtracted. The concentration of MDA was calculated using its extinction coefficient of 155 mM/cm.

Formula to calculate Concentration of MDA:

$A = \varepsilon c l \Rightarrow c = A / \varepsilon l$

Where:

A – Absorbance,

ε - Molar Extinction coefficient / Molar Absorptivity,

c – Concentration of MDA (mM),

l – Path length (cm) (= 1cm)

1.7 Chlorophyll Content Estimation (before application of the extract).

300mg of leaves are cut into small pieces and ground after the addition of liquid nitrogen. 5ml of 80% acetone is then added to a 15ml falcon tube and the powder is transferred into it. The mixture is then mixed in the dark for 15-30 minutes. This step is followed by centrifugation at 4˚C for 15 minutes at 3000 rpm and the supernatant is transferred to a new centrifuge tube and kept in the dark. This step maybe repeated if necessary. The supernatants are then mixed and absorbance of the mixture is measured using a spectrophotometer. The concentration of chlorophyll in the leaves is then calculated as follows:

Chlorophyll a (mg/g) = $(12.7 \times A_{663} - 2.69 \times A_{645}) \times V/1000 \times W$

Chlorophyll b (mg/g) = $(22.9 \times A_{645} - 4.86 \times A_{663}) V/1000 \times W$

Chlorophyll a+b (mg/g) = $8.02 \times A_{663} + 20.20 \times A_{645} \times V/1000 X W$

where

A= Absorbance,

V=Volume of extract (ml),

W=Weight of leaves (g).

30 ml extract of 30 mg/ml concentration was sprayed on the field on parthenium plants. Calculations were made using the formula $C_1V_1=C_2V_2$.

1.436 ml of the concentrated extract (626.5 mg/ml) extract was taken and the volume was made up to 30 ml and used as the herbicide.

2. RESULTS AND DISCUSSION

Purification of the Extract

The pomegranate peel extract obtained after concentration was purified using TCA Acetone method in order to remove any protein present in the extract that may hinder its herbicidal efficiency. No precipitate was obtained after centrifugation indicating that the extract contained very little or no protein in it. Thus, this step can be omitted in the process of herbicide formulation. This also increases the cost effectiveness of the herbicide as the omission of the purification step reduces the number of chemicals required as well as time required for the development of final extract.

Germination Studies

One of the most common methods of evaluating the herbicidal efficiency of a material is its ability to inhibit germination of the target species. 4 most common weeds that threaten farmlands in India, *Galinsoga parviflora*, *Parthenium hysterophorus*, *Tridax procumbens* and *Spermacoce ocymoides* were chosen for this study. The seeds of these weeds were germinated with the aid of the concentrated pomegranate peel extract at concentrations of 5 mg/ml, 10 mg/ml, 15 mg/ml, 20 mg/ml, 25 mg/ml and 30 mg/ml with germination of seeds using water as control. The percentage of germination inhibition was calculated using the formula mentioned before. The percentage of germination inhibition was found to be highest for parthenium and false button weed which gave a complete inhibition at 5 mg/ml, followed by gallant soldier that gave a complete inhibition at 30 mg/ml. At 5mg/ml the herbicide seemed to promote the growth of gallant soldier. This indicates that pomegranate peel extract may behave as a fertilizer as low concentrations but acts as a herbicide at higher concentrations as all the weeds showed 100% germination inhibition at 30mg/ml. Tridax did not show any visible shoot or root production. Increased pigmentation around the seeds was observed with increasing concentration of herbicide indicating some notable effect.

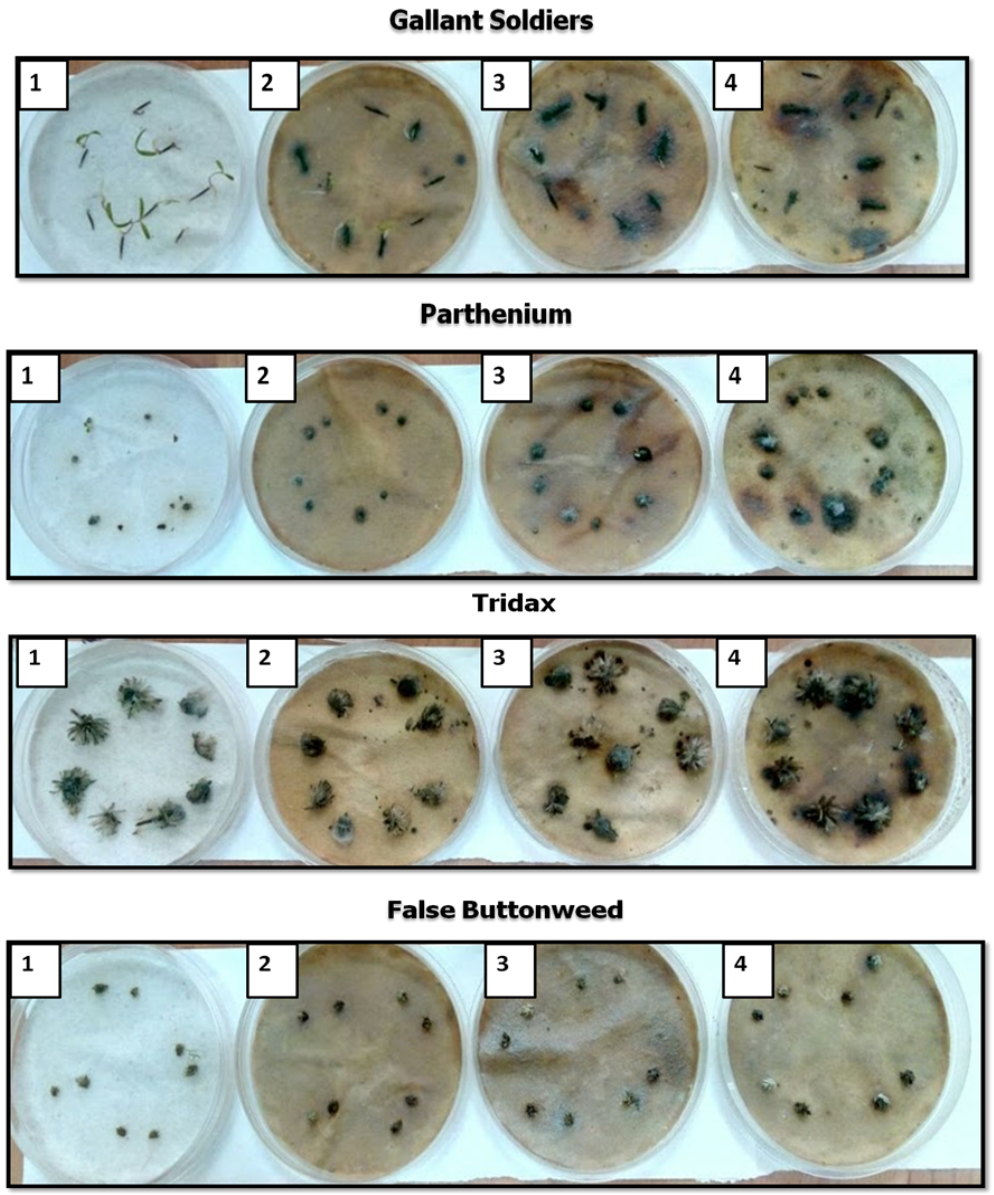
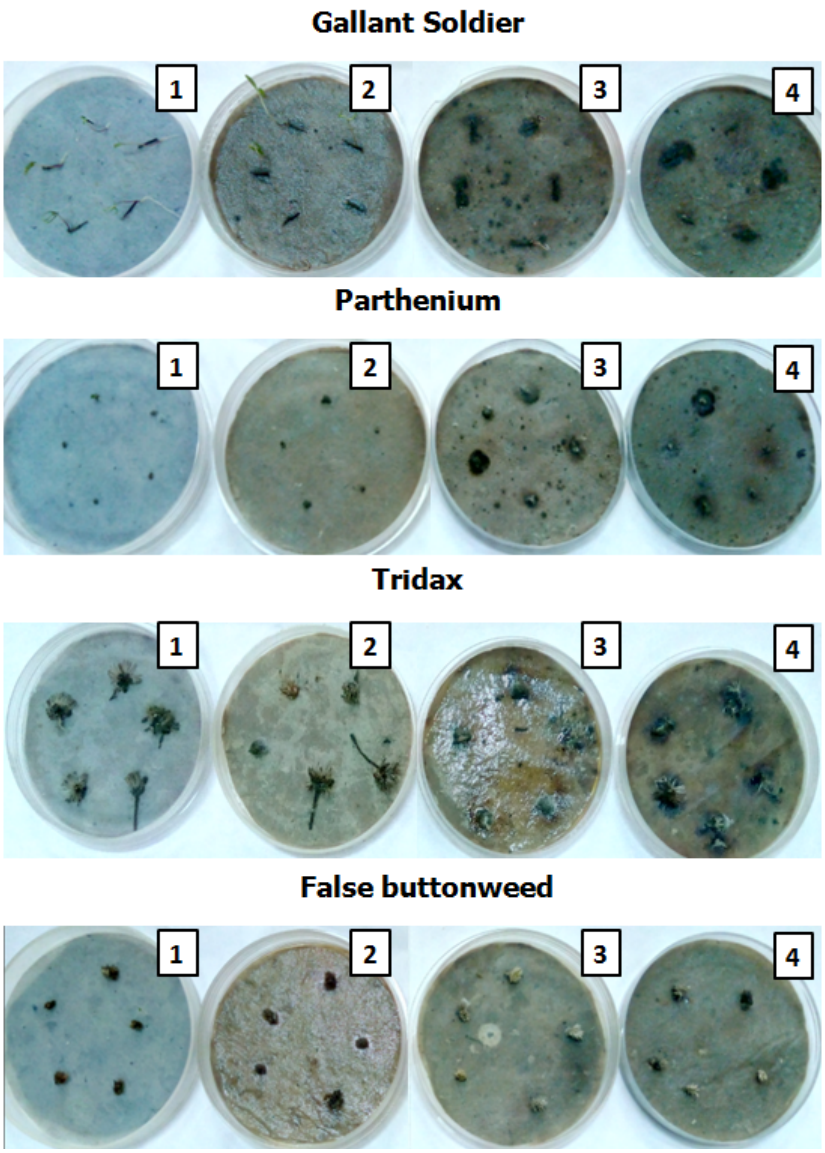


Figure 1: Germination Plates. Control has seeds of the 4 common weeds that inhabit India grown with the aid of water (1) while the test plates consist of the same seeds grown with the extract of concentrations 10mg/ml, 20mg/ml and 30mg/ml respectively (2, 3 and 4).

Table 1: Consolidated results for germination studies using herbicide at 10mg/ml, 20mg/ml and 30mg/ml. The highest herbicidal activity was found against parthenium and false button weed that gave a complete inhibition at 10mg/ml, followed by activity against gallant soldier that gave a complete inhibition at 30 mg/ml.

	Length of shoot in (cm)				**% Germination inhibition for**		
PLANT	**CONTROL**	**10mg/ml**	**20mg/ml**	**30mg/ml**	**10mg/ml**	**20mg/ml**	**30mg/ml**
Gallant Soldiers	2.5	1.5	0.5	0	40	80	100
Parthenium	0.3	0	0	0	100	100	100
Tridax	--	--	--	--	0	0	0
False Buttonweed	1.5	0	0	0	100	100	100



1- Control, 2- 5mg/ml, 3- 15 mg/ml, 4- 25 mg/ml

Figure 2: Germination Plates. Control has seeds of the 4 common weeds that inhabit India grown with the aid of water (1) while the test plates consist of the same seeds grown with the extract of concentrations 5mg/ml, 15mg/ml and 25mg/ml respectively (2, 3 and 4).

Table 6.2.2: Consolidated results for germination studies using herbicide at 5mg/ml, 15mg/ml and 25mg/ml. The highest herbicidal activity was found against parthenium and false button weed that gave a complete inhibition at 5 mg/ml, followed by activity against gallant soldier that gave a complete inhibition at 25 mg/ml.

Plant	**Length of shoot in control (cm)**	**Length of shoot in 5 mg/ml extract (cm)**	**Length of shoot in 15 mg/ml extract (cm)**	**Length of shoot in 25 mg/ml extract (cm)**	**% Germination inhibition for 5 mg/ml**	**% Germination inhibition for 15 mg/ml**	**% Germination inhibition for 25 mg/ml**
Gallant Soldier	1.7	2.4	0.8	0	-41.1764	52.9411	100
Parthenium	0.5	0	0	0	100	100	100
Tridax	-	-	-	-	0	0	0
False button weed	0.7	0	0	0	100	100	100

SEM Analysis

SEM analysis was carried out in order to study the surface morphological changes after the application of the herbicide. The shrinking of chloroplasts with the increase in action of herbicide was aimed to be recorded but chloroplasts were unable to be visualized, thus the study was shifted to observation of structural changes of stomata. As the action of herbicide increases, the leaves begin to dry up and stomata open up widely. Images of the surface structure of each leaf as well as the stomatal structures before the application of the pomegranate peel extract were recorded.

Weed 1 (Before application of the extract)

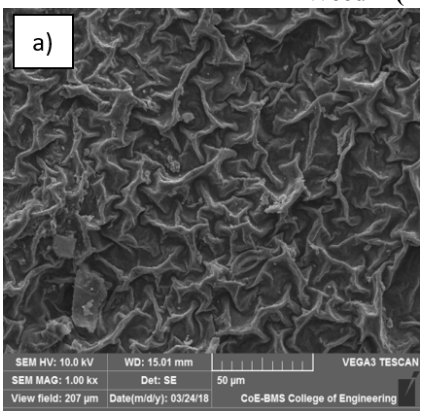


Figure 3 a) Surface morphology of a leaf of Weed 1.

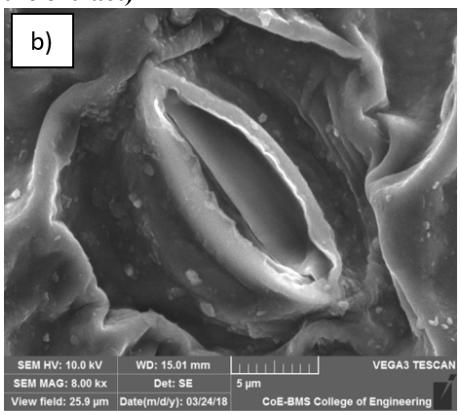


Figure 3 b) Stomata in the leaves of Weed 1.

Weed 2 (Before application of the extract)

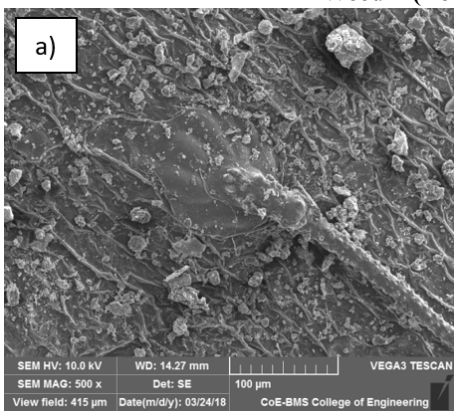


Figure 4 a) Surface morphology of a leaf of Weed 2.

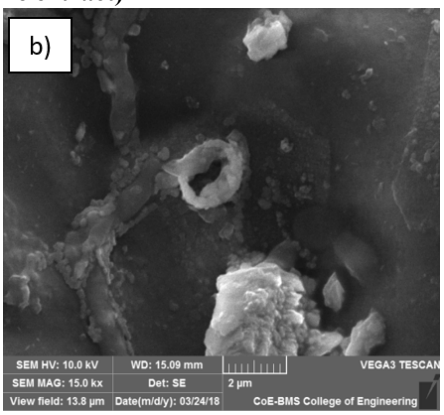


Figure 4 b) Stomata in the leaves of Weed 2.

Weed 3 (Before application of the extract

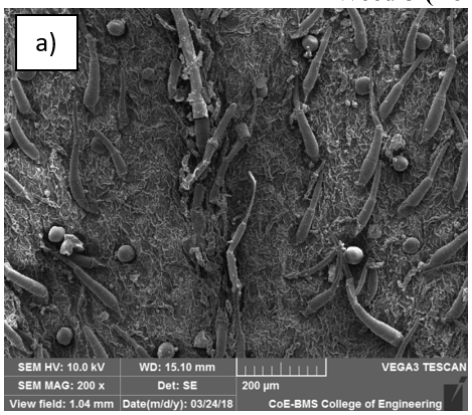


Figure 4 a) Surface morphology of a leaf of Weed 3.

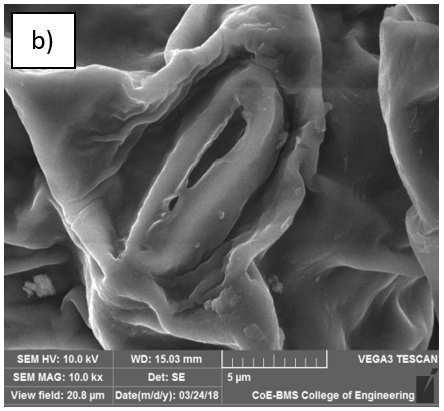


Figure 4 b) Stomata in the leaves of Weed 3.

Weed 4 (Before application of the extract)

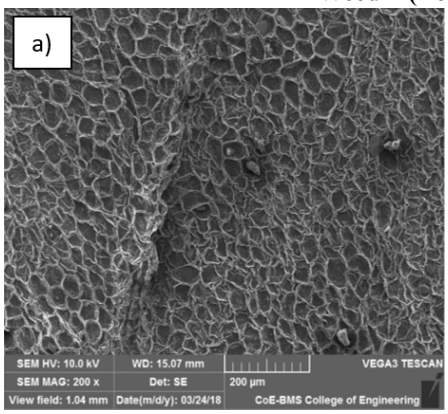


Figure 4 a) Surface morphology of a leaf of Weed 4.

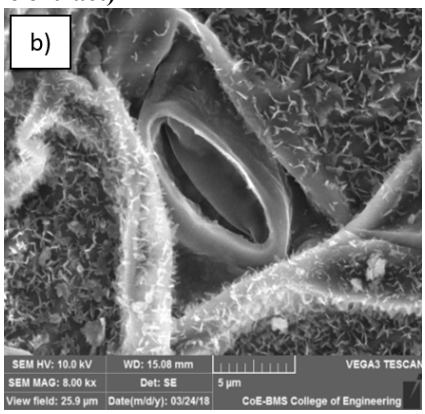


Figure 4 b) Stomata in the leaves of Weed 4.

Weed 5 (Before application of the extract)

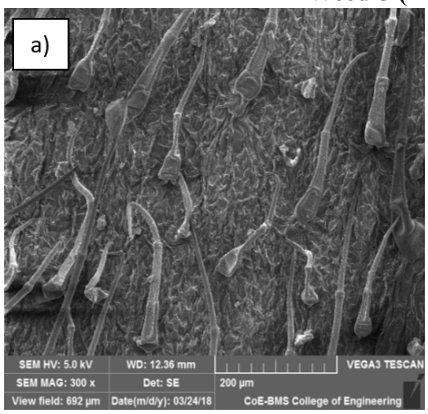


Figure 5 a) Surface morphology of a leaf of Weed 5.

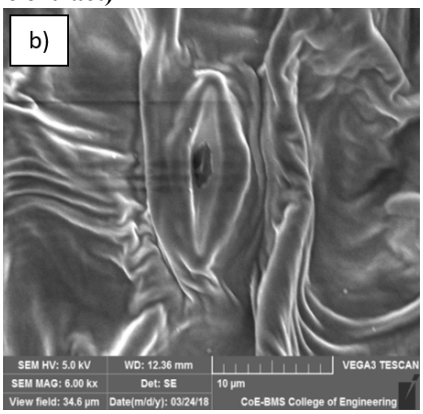


Figure 5 b) Stomata in the leaves of Weed 5.

Chilli Leaf (Before application of the extract)

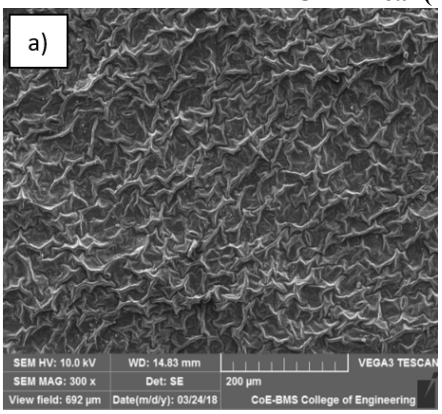


Figure 6 a) Surface morphology of a leaf of Chilli leaf.

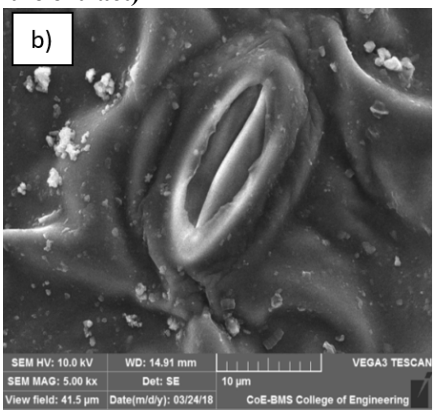


Figure 6 b) Stomata in the leaves of Chilli leaf.

Total Phenol Quantification

One of the native species in the field chosen was chilli plant as shown in figure 6.4.1. The application of extract can affect the uptake kinetics of the plant. Uptake kinetics can be calculated using total phenol quantification by constructing a gallic acid standard curve of different concentrations. The method followed was Folin-Ciocalteau reagent method for calculating the total phenol concentration before the application of the extract on the plant.

Gallic acid standard curve was prepared using increasing concentrations of Gallic acid from a stock solution of 20µg/ml. Simultaneously the chilli plant leaf was macerated in 1ml of water and used for testing. Increasing intensity of colour was observed with increasing concentration of Gallic acid.

Table 2: Absorbance values of increasing concentrations of Gallic acid and absorbance values of the chilli plant leaf from the site chosen obtained after Folin-Ciocalteau method

of determination of total phenol content. The assay was carried out in duplicates and the mean of the absorbances obtained was used for plotting the graphs.

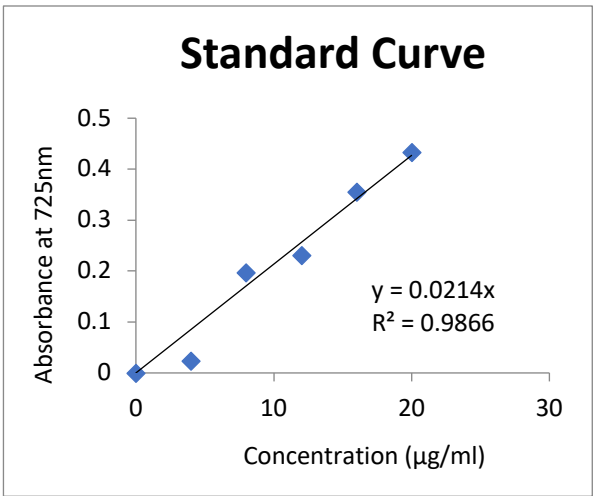
CONC (µg/ml)	OD1	OD2	OD mean
0	0	0	0
4	0.023	0.023	0.023
8	0.156	0.238	0.197
12	0.217	0.245	0.231
16	0.319	0.392	0.355
20	0.403	0.463	0.433
?	0.210	0.392	0.301

Unknown Concentration $(x) = \frac{y}{m}$

= (0.301*2)/0.021

= 28.667 µg/ml

Gallic acid standard curve was obtained by plotting concentration on the X axis and absorbance measured at 725 nm on Y axis. Unknown concentration of the chilli leaf was found using the linear equation obtained. A linear equation y= 0.021x was obtained for the Gallic acid standard curve with a regression factor of 0.963. The total phenol concentration of the chilli plant leaf was found to be 28.667µg/ml.



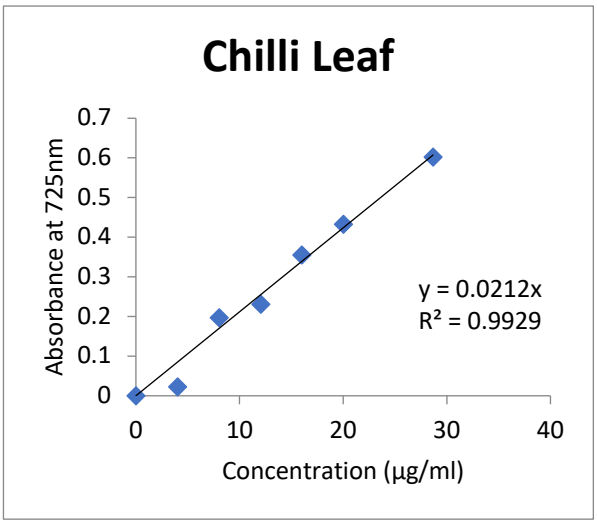


Figure 7 Gallic acid standard curve with a linear equation of y = 0.021x and a regression factor of 0.963. For the chilli plant leaf, obtained linear equation was the same as the standard curve and a regression factor of 0.980.

Characteristics of Herbicide

The characteristics of an herbicide are important to determine the formulation, the effectiveness of the usage, dosage of the herbicide and many other factors that are needed to assess the profitability of the herbicide for the farmers. The formulation characteristics according to CIPAC method formulation are such as suspensibility, wettability and pH. Suspensibility is defined as the amount of active ingredient suspended after a given time in a column of liquid, of stated height, expressed as a percentage of the amount of active ingredient in the original suspension. Wettability is the tendency of one fluid to spread on, or adhere to, a solid surface in the presence of other immiscible fluids.

Suspensibility – Weight of the suspension was calculated to be **0.0633 g**.

Supension Stability is given by the percentage suspensibility. The formula is as follows:

Suspensibility % $= \frac{10}{9} \times \frac{m_1 - m_2}{m_1} \times 100$

where m_1 is the pesticide content in original suspension and m_2 is the pesticide content in the remaining solution in the cylinder.

Calculations:

$m_1 = 0.625g$ and $m_2 = 0.0633g$

Suspensibility % = 99.857

Wettability – Time recorded for the lyophilized extract to disperse in distilled water was **9 seconds**.

pH Range – Measured pH of the extract was found to be **3.4**.

Lipid Peroxidation

Lipid peroxidation refers to the oxidative degradation of lipids. Quantification of lipid peroxidation is essential to assess oxidative stress in pathophysiological processes. Antioxidant property of phenolic compounds leads to excessive lipid peroxidation and eventually the damage of tissues. Therefore, it becomes necessary to evaluate this activity before and after application of the extract on the weeds and the beneficial plant chosen.

The assay was carried out using 2-Thiobarbituric acid (TBA) as a substrate which reacts with malondialdehyde (MDA) and forms a complex that is measured spectrophotometrically at 532nm. The absorbance at 600nm is measured for non-specific absorption. The concentration of MDA is calculated using its extinction coefficient of 155mM/cm. The experiment was done in duplicates.

The formula for calculating the concentration of MDA (mM) is as follows:

Formula:

$$A = \varepsilon c l$$

Where,

A – Absorbance

ε - Molar Extinction coefficient / Molar Absorptivity

c – Concentration of MDA (mM)

l – Path length (cm) (= 1cm)

Calculation:

$$c = \frac{A_{532} - A_{600}}{155} \text{ (mM)}$$

Table 3: Absorbances at 532nm and 600nm (mean) and the concentration of MDA (mM) calculated for each of the five weeds and chilli plant leaf before the application of the herbicide. The concentration of MDA was found to be the highest in Weed 3, followed by Chilli leaf, which is followed by Weed 1, Weed 5, Weed 4 and finally Weed 2 which showed the least concentration of MDA.

Sample	A 532nm (1)	A 532 nm (2)	A 532nm (Mean)	A 600nm (1)	A 600nm (2)	A 600nm (Mean)	Final Absorbance ($A_{532} - A_{600}$)	Conc. of MDA (mM)
Weed 1	0.022	0.053	0.0375	0	0.01	0.005	0.0325	0.000209677
Weed 2	0.02	0.022	0.021	0	0.019	0.0095	0.0115	7.41935E-05
Weed 3	0.099	0.121	0.11	0.066	0.063	0.0645	0.0455	0.000293548
Weed 4	0.029	0.043	0.036	0.012	0.026	0.019	0.017	0.000109677
Weed 5	0.022	0.034	0.028	0	0.016	0.008	0.02	0.000129032
Chilli leaf	0.034	0.048	0.041	0	0.001	0.0005	0.0405	0.00026129

Chlorophyll content estimation

Chlorophyll is a pigment essential for the survival of plants. It helps in regulation of photosynthesis which is in turn essential for the growth as well as survival of plants. As the chlorophyll content in the plant reduces, the rate of photosynthesis comes down, eventually leading to the death of the plant. Thus chlorophyll content forms an important parameter to monitor the efficiency of an herbicide. The chlorophyll content is hypothesised to reduce after the application of the herbicide.

The chlorophyll content present in each of the samples was quantified by colorimetric assay and the values were obtained by using the formulae mentioned before. The highest amount of chlorophyll content was found in Weed 5 with a total chlorophyll content of 0.063 mg/g of leaves, followed by weed 3 with a total chlorophyll content of 0.058 mg/g of leaves, followed by Weed 1 with a total chlorophyll content of 0.011 mg/g of leaves, followed by Weed 2 and Chilli which had a total chlorophyll content of 0.007 mg/g of leaves. Figure 2 shows the test tubes with the final reaction mixture obtained. Higher degree of colouration indicates higher concentration of chlorophyll content. The absorbance obtained at 663 nm and 645 nm respectively of each weed sample chosen as well as native plant (chilli) and the chlorophyll content present in each of them is shown in table 4. The assay was carried out in duplicates.

Table 4: Absorbance at 663 nm and 645 nm as well as chlorophyll content present in each weed sample and native plant (chilli) chosen before application of herbicide. Weed 5 showed maximum chlorophyll content followed by Weed 3, followed by Weed 1, followed by Weed 2 and finally Weed 4 and Chilli which had the same amount of chlorophyll content.

Plant	Weight (g)	Final Volume (ml)	$A_{663\ 1}$	$A_{663\ 2}$	A_{663} mean	A_{6451}	A_{6452}	A_{645} mean	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Total chlorophyll content (mg/g)
Weed 1	0.074	2.48	2.966	2.942	2.954	1.788	1.635	1.712	0.06	0.046	0.011
Weed 2	0.067	2.22	3	3.096	3.048	1.811	2.121	1.966	0.05	0.045	0.01
Weed 3	0.19	6.32	2.889	2.721	2.805	1.413	1.133	1.273	0.387	0.186	0.058
Weed 4	0.146	4.86	0.735	0.67	0.703	0.204	0.184	0.194	0.06	0.007	0.007
Weed 5	0.19	6.32	2.95	2.778	2.864	1.674	1.244	1.459	0.39	0.234	0.063
Chilli	0.065	2.16	2.964	2.01	2.487	1.754	1.091	1.423	0.039	0.029	0.007

Effect of Spray on the Parthenium plants in the chosen field

Gradual drying up of the leaves was observed after spraying of the extract as shown in Figure 8. This shows that the extract does have herbicidal properties. The herbicidal activity may be due to the high concentration of phenolic compounds present in the extract. A second spray may be required for complete drying up of the plant. Further studies are being carried out to optimize the concentration of herbicide for the other weeds chosen.

Figure 8: Gradual drying up of the plant over 5 days indicating gradual death of the plant. This shows that the extract does possess herbicidal activity.

CONCLUSION

Pomegranate peel seemed to show very good herbicidal properties on chosen weeds. The chlorophyll content in these plants decreased after the application of the herbicide, while lipid peroxidation increased. Therefore, the probable mechanism of action of the herbicide is by chlorophyll degradation and oxidative cell damage. Further studies are required to analyse other weeds and establish a probable set of target weeds for the herbicide. Germination studies proved that pomegranate peel extract establishes complete inhibition of germination. Soil biota studies also highlighted that the herbicide developed did not have a significant negative impact on soil biota implying that it is eco-friendly and safe. Since pomegranate peel is organic, the herbicide is biodegradable and having less chances of bioaccumulation. The herbicide developed also overcomes other limitations of synthetic herbicides due to it cost effective nature. Specificity of the herbicide to weeds was also established to a certain extent. This makes the spraying of herbicide easy and reduces the loss of yield of beneficial crops for the farmers, thus increasing their revenue. Future studies suggest to analyse of herbicide resistance over the years and identifying the group of weeds and beneficial species that the extract is specific to. Some studies also showed fertilizer effect of the herbicide at low concentrations, therefore finding the optimum concentration of the extract required to eradicate the different types of weeds remains a challenge.

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