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INFLUENCE OF SIMULATED ACID RAIN ON MORPHOLOGICAL AND PHYTOCHEMICALS ATTRIBUTES OF GUAR (*CYAMOPSIS TETRAGNOLOBA* L.)

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Abstract

The global environment is contaminated day by day due to urbanization, industrialization, burning of fossil fuels, etc., causing the occurrence of toxic gases in the environment. These gases are mixed with wet air and cause acidic rain, which can foremost be hazardous to plant kingdoms, reduction of crop yield, forest declination, and acidification of water and soil.

Guar *(Cyamopsis tetragonoloba* L.) is a leguminous plant that is mainly cultivated as an important commercial and export crop of Pakistan. The aim of the present study is to examine the effect of simulated acid rain (SAR) of various ranges of pH of HNO_3 on morphology and phytochemicals attributes of Guar due to very limited information available on the harmful effects of acid rain on different crops, especially on Guar. The results of existing study showed that the growth of Guar was remarkably inhibited by SAR of pH 2.0 and pH 3.0. However, the number of leaves and shoot were appeared but badly influenced when treated with SAR solution of pH 4.0 and pH 5.0. Whilst, the number of leaves, shoot length, and number of fruits increased at pH 6.0.

A comparative study of the phytochemical investigation of SAR-affected plants, exhibited high total protein contents, total sugar, phenolic compounds, and antioxidant activity at pH-6 though reduced at low pH 4.0 and 5.0 of HNO_3 used as SAR. It is concluded that the growth and phytochemicals ingredients at low pH were badly affected and highly reduced as compared to the control.

Key words: Guar, Acid rain, pH, Morphological characters, Phytochemicals.

Introduction

Industrialization causes a destructive impact on our environment due to the discharge huge number of gases such as sulfur dioxide (SO_2), carbon monoxide (CO) and nitrogen oxides (NO_2) are the main motives of air pollution released from industrial sources, burning of fossil fuels and garbage waste. These gases are converted into sulfuric (H_2SO_4) and nitric (HNO_3) acids in precipitation, which eventually causes Acid Rain (A.R) (Kita *et al*., 2004). These pollutants don't remain in a specific area and might be transferred to another place and disturb all parts of ecosystems (Alrawi, 2022; Dubey, 2013). Over the past two eras, A.R has developed a cumulative problem in Asia (Debnath *et al*., 2018a). Plants are vital elements of ecosystems and are influenced by A.R pollution (Liu *et al*., 2018a). Acid Rain disrupts the stem, leaves, and roots of plants (Huang *et al*., 2019) and also decreases the germination, photosynthesis, and chlorophyll contents of the plant (Liu *et al*., 2018b) and hence declines crop yields (Liao *et al*., 2005).

Previously investigated A.R disturbs the growth of plants directly and indirectly. The direct destruction of AR to foliage of plants contains physiological and morphological injuries (Kohno 2017; Du *et al*., 2017) as well as also influences some essential phytochemicals (tannin, phenolics, flavonoids and saponins), which are feasibly beneficial for human beings (Garcia-Salas *et al*., 2010).

Guar (*Cyamopsis tetragonoloba* L.) belongs to the family of Leguminosae (Fabaceae), a highly important upright, deep-rooted plant due to its greatest yield, drought tolerance and best nutritional value. Guar is deliberated as a remarkable crop of Pakistan (Mudgil *et al*., 2014) and yield approximately 70,000 tons of Guar yearly (Sultan *et al*., 2013). Pakistan exported 25800 tons of Guar and its based products to 30 foreign countries and grossed about 2206400 rupees as a foreign exchange during 2008-2009 (Anon., 2009). Though, the cultivation yield of guar in the country as compared to some developed countries of the world remained short due to variations of environmental conditions containing pollution caused by A.R. In the geographical region, Pakistan is situated in arid and semi-arid regions. About 80% of Pakistani people are reliant on horticulture (Jatoi, 2020). It is doubt in future, the chances of acid rain occurring in adjoining Thar areas of Sindh, Pakistan where the Thar coal project is in progress and industrialization is also going on rapidly. The local people of Thar mostly depend on rain water for crop cultivation. The importance and need of exploring to observe the effects of simulated acid rain on different crops specially Guar to avoid its harmfulness and may also helpful for awareness to farmers by prior planning if acid precipitation occurs in the environment. Due to keeping in view the impotence of Guar which is main crop of Thar and protects it from future threats of acid rain, the present study is designed to investigate the outcome of Simulated Acid Rain (SAR) *In vitro* on morphology and phytochemical contents of Guar.

Material and Method

The present study was conducted on the Guar plant (*Cyanosis tetragonoloba* L.). The certified seeds of Guar were obtained from National Agricultural Research (NARC) Islamabad, Pakistan. The five (05) seeds of guar plant were germinated on each pot containing loamy soil and kept in Green House with replicates. The SAR solutions were made by Nitric acid of various pH levels (2, 3, 4, 5 and 6) as reported by (Jacobson *et al*., 1987). Each seedling plants were sprayed (40 ml) with pH solution of SAR after growth of one month subjected to simulated acid rain (a specific period). At the late afternoon a spray applied light intensity of 25-45% at noon. Sprays of SAR were used one time in a week and six weeks later the volume of spray was doubled in a week. The Guar plants were harvested after the exposure of SAR and collected the different segments of *Cyamopsis tetragonoloba* L. plant (stem, leaves and fruit), weighed, washed and dried for one week at room temperature till the water contents removed completely.

Different phytochemicals / biochemicals were analyzed quantitatively and qualitatively from 20% various parts (leaves, fruit, and stem) of *Cyamopsis tetragonoloba* L. extracts.

Quantification of total sugar, reducing sugar, total protein contents: The total sugar and reducing sugar contents were determined from 20% different parts of *Cyanosis tetragonoloba* L. (aqueous and ethanol) extracts using glucose as a standard curve by reported (Montgomery 1961; Miller 1959) methods respectively. The reaction mixture for total sugar contained 0.5 ml test solution along with 2.5 ml H_2SO_4 and 50 µl 80% phenol, read the absorbance at 485 n.m. However, reducing sugar contents estimated from reaction mixture consisted of the 1ml test sample and 1ml dinitrosylicylic acid (DNS) reagent and color intensity read at 540 nm by spectrophotometer. Whilst, total protein from 20% extracts (ethanol and aqueous) of various segments of *Cyamopsis tetragonoloba* L. were quantified by (Lowry *et al*., 1951) method. 0.5 ml test sample and 2.5 ml of the alkaline copper reagent mixed thoroughly and retained at room temperature for 10 minutes then added 0.25µl Follin-Ciocalteu reagent (1:1 v/v with distill water). The absorbance was read against reagent blank at 750 nm by UV-visible spectrophotometer. The concentration of total protein was calculated by the standard curve of bovine serum albumin.

Quantification and qualitative analysis of phytochemicals: The amount of total antioxidant activity was quantified by spectrophotometer from 20% extracts (aqueous and ethanol) of different parts of *Cyamopsis tetragonoloba* L. prescribed by (Prieto *et al*., 1999) using α-Tocoferol as standard curve. The test sample (0.2 ml) combined with 2 ml of reagent assorted of 0.6 M H_2SO_4, 28 mM sodium phosphate and 4mM ammonium molybdate. The reaction mixture containing tubes were capped with aluminum foil and incubated in the boiling water bath at 95°C for 90 minutes then cooled at room temperature. The optical density noted at 695 nm against the reagent blank.

While, (Yasoubi *et al*., 2007) method was followed for quantification of phenolic compounds from 20% *Cyamopsis tetragonoloba* L. (aqueous and ethanol). In this regard, 0.2 ml test sample mixed with 1.0 ml of 10-fold diluted Folin-Ciocalteu reagent and 0.8 ml of $NaCO_3$. The reaction mixture was conserved at room temperature for 30 minutes and then read the absorbance at 765 nm by UV-visible spectrophotometer. The reading of the absorbance was calculated by a standard curve, drawn by different concentrations of Gallic acid.

However, some different phytochemicals were also analyzed qualitatively like Alkaloids, Flavonoids, and Tannins by their respective reported methods (Soni & Sosa, 2013; Njoku *et al*., 2011; Rahu *et al*., 2021) from extracts of *Cyamopsis tetragonoloba* L. plant.

Statistical Analysis

The data of experiment have been existed as the mean ± SD (Standard deviation) of three replicates. The hypothesis has been examined by t-test. Variances were calculated as significant at $p<0.05$.

Results

Guar (*Cyamopsis tetragonoloba* L.) is an annual legume crop mostly growing in Pakistan and other continents. Guar plant is the main source of protein content of food. It is used as fodder, food and medicinal purpose. The present research objectives and purpose are: To analyze the morphological impact of the simulated acid rain applied to the plant *Cyamopsis tetragonoloba* L. and evaluate its effects on phytochemicals / biochemicals of under study plant.

The SAR treated plants were observed on the basis of different parameters like impact of simulated acid rain on morphological and phytochemicals of Guar plant. The data recorded for various numerical parameters were statistically analyzed as per the standard procedure.

In the present study, 05 healthy seeds were germinated in each pot. The sowing seeds were applied with a spray of SAR of different pH of HNO_3 and observed its effect on plant seeds.

Impact of simulated acid rain of different pH of HNO_3 on germination of seeds

pH-2.0: Germination of the seeds of Guar completely inhibited (0%) when SAR of pH-2.0 (HNO_3) was applied.

pH-3.0: The Guar seeds started to germinate about 54% after 10 days at pH-3.0 of SAR was applied but their radical and germinated shoots emerged to be declined and thin.

pH-4.0: The Guar plants' seeds began to germinate after 13 days and 66% of seeds germinated and the other remaining were affected by simulated acid rain of pH-4.0 (HNO_3).

pH-5.0: After 12 days of germinations, 74% seeds were germinated when SAR of pH 5.0 were sprayed and also observed some radical germination, shoot emerged and within a few days leaves also appeared in some seeds.

pH-6.0: 80% seeds germination rate was recorded when Guar plant seeds were showered with simulated acid rain of pH 6.0. The resulting of SAR treatment of pH 6.0, radical, shoots and leaves were appeared within 10 days.

Seeds germination in control: Seeds began to germinate after 08 days, and all seeds were completely germinated as presented (Table 1; Fig. 1).

Impact of simulated acid rain of different pH of HNO_3 on morphological characters of Guar plants

Impact of pH 2.0: However, after 30 days of plant age, the SAR of pH 2.0 (HNO_3) showered on the guar plant, the growth of Guar plant inhibited, no node occurred within 40 days. The plants' leaves curled, their size were reduced; increased in leaves gaps and about 15-18 leaves appeared. Following 42 days, the leaves became yellowish, resulting in chlorosis and abscission of the leaves. The stem tips rotted, twisted, wrinkled and delicate, as new leaves emerged but in a curled position. Necrosis appeared on the leaves within a couple of days. During the first week of plant exposure to simulated acid precipitation, the plant started to die. It was observed that pH 2.0 level is very acidic for plant germination as well as for growth and plants were destroyed within 45 days in comparison to control in which 100% of seeds were germinated within 08 days as exhibited (Table 2; Fig. 2).

Impact of pH 3.0: When the SAR of pH 3.0 technique was applied on Guar plants, the growth of the plant stunted. A thin bristle stem possesses no branch and weak stem erected. The gap of leaves between two nodes increased; hence there would be the number of leaves decrease. Some leaves appeared but showed necrosis on leaves, and also yellowish spots appeared probably. The leaves which were older happening fall off and early leaves abscission started. The new leaves emerged curly in shape. The edges of leaves rotted, wrinkled and curled. Around 48 days there was no flower appeared and the plant completely vanished as depicted (Table 2 & Fig. 2).

Impact of pH 4.0: After plant maturation, SAR of pH 4.0 spray has affected the growth of plants. The stem became soft and thin. The branch has not been emerged; the size of shoot was noted about 20 to 22 cm, in which leaves can be counted easily and about 38 to 40 leaves on each plant appeared as exhibited in Table-2 and Figure-2. However, the leaves were curved, thus enhanced the leaf gap between two nodes. Chlorosis existed when leaves converted into yellowish older leaves, turned into curly shape and immediately falling off and also new leaves tips became burning and wrinkling.

Impact of pH 5.0: SAR of pH 5.0 was sprayed on plants after 30 days showed some signs of slow growth, fragile stem, and soft erection and leaves gapes of two nodes whilst, the number of leaves was slightly increased approximately 46 leaves as well as shoot and root length also increased vaguely along with 2 to 3 branches as compared to SAR of pH 4.0 treated plants. Affected leaves probably dropped out young leaves grown in flexible positions, older leaves edges decaying, discoloration and curling and leaves abscission began. There was only one branch that occurred while no flower existed at pH 5.0 as summarized in Table 2 and Fig. 2.

Impact of pH 6.0: The results of the impact of SAR of pH 6.0 (HNO_3) showered on Guar plants was obtained surprisingly better than SAR of pH 4.0 and 5.0 treated guar plants but less as compared to control. The growth of plants was stunted after applying SAR to plants. Only four branches on stems appeared. However, the branches were weak and leaves also affected and the distance between the nodes was increased. In June-2021, only two flowers appeared after 70 days. It was normal for vegetative growth and plants to survive. After 80 days some more flowers and fruits appeared. On the other hand, control plants were grown normally and healthy and plants can be seen with broad stems and several branches. The number of leaves was increased about 80 to 90. The first flower developed at the end of September-2021. The plants survived and gave the fruit to many flowers. The obtained results showed the effects of SAR (HNO_3) on Guar plant maturation as presented (Table 2; Fig. 2).

Quantification of total sugar from 20% aqueous and ethanol extracts of SAR treated Guar plants: Total sugar was evaluated from the aqueous and solvent extract of the Guar plant. The result of total sugar (Fig. 3) indicated that the maximum range in 20% aqueous extracts of different segments of Guar plants, showered with pH 6.0 of HNO_3 solutions used as simulated acid rain (SAR) was calculated as fruit; 3.061 mg/ml, leaves 1.985 mg/ml and 1.709 mg/ml in stem respectively. Whereas the lowest amount of total sugar was noted as compared to pH 6.0 was recorded in stem aqueous extract at pH 4.0 (0.649 ± 0.043 mg/ml) and aqueous extract of leaves (1.709 ± 0.152mg/ml). Furthermore, it was observed that the highest concentration of total sugar was calculated in control plants (without treated SAR) than all treated Guar plants with different pH solutions of nitric acid.

Table 1. Impact of simulated acid rain of different pH of HNO_3 on germination of Guar.

Parameter	pH 2	pH 3	pH 4	pH 5	pH 6	Control
Root	-	0.6 cm	0.8 cm	0.9 cm	1.0 cm	1.9 cm
Shoot	-	1.3 cm	1.5 cm	1.8 cm	1.9 cm	2.0 cm
Leaves	-	0.3 cm	0.4 cm	0.5 cm	1.0 cm	1.7 cm
Germination %	-	54%	66%	74%	80%	100%

Table 2. Effect of simulated acid rain of different pH (HNO_3) on different morphological characters of *Cyamopsis tetragonoloba* L. plants.

Parameter	pH 2.0	pH 3.0	pH 4.0	pH 5.0	pH 6.0	Control
Shoot length (cm)	---	---	22.05	26.08	31.6	60.73
Root length (cm)	---	---	18.10	24.52	26.58	27.73
Number of leaf	---	---	40	46	58	90
Number of branches	---	---	---	3	4	8
Number of flowers	---	---	----	---	10	15
Number of fruits	---	---	---	---	8	14

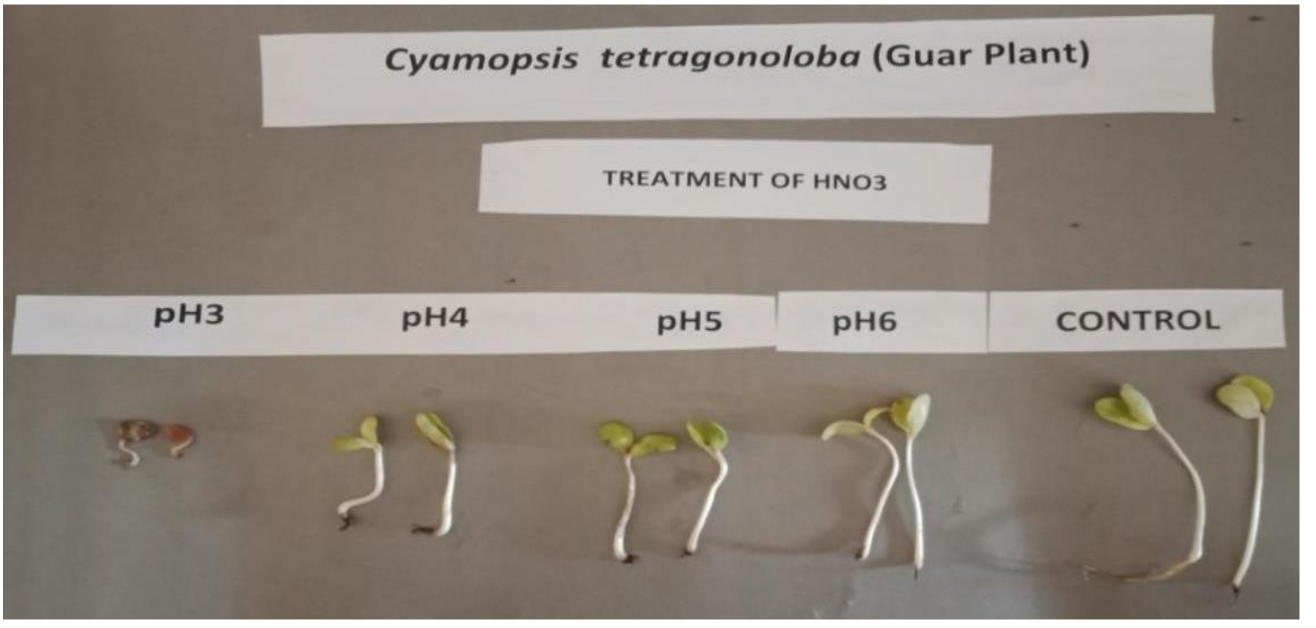

Fig. 1. Comparison of Seed germination, after applying SAR treatment of different pH of HNO_3.

Fig. 2. Impact of Simulated Acid Rain of Nitric Acid after maturation of the plant.

Total sugar was also investigated from the ethanol extract of the Guar plant but results were not different as obtained in aqueous extracts of Guar plants sprayed with SAR and also low as compared to aqueous extracts. The maximum quantity of total sugar was estimated in fruit ethanol extracts at pH 6.0 (2.26 ± 0.16 mg/ml), ethanol leaves extracts (1.96 ± 0.05 mg/ml) at pH 6.0 level, whereas the minimum quantity of total sugar was exposed in stem ethanol extracts at pH 4.0 (0.526±0.086 mg/ml) and leaves ethanol extract (1.54 ± 0.04 mg/ml) correspondingly. However, these results of SAR treated plants are comparatively low than control grown plants without any addition of pH solution of HNO_3 used as SAR as presented in (Fig. 4).

Quantification of reducing sugar from 20% aqueous and ethanol extracts of SAR treated Guar plants: The reducing sugar contents were also examined from 20% extracts of different parts of *Cyamopsis tetragonoloba L.* The greatest amount of reducing sugar results was obtained but less than control (without treatment of SAR) at pH 6.0 from aqueous extracts of different parts (Fruit, leaves and stem) of Guar plants 0.012 ± 0.0002 mg/ml, 0.009 ± 0.0004 mg/ml and 0.008 ± 0.0006 mg/ml accordingly. While the minimum quantity of reducing sugar was measured at pH 4.0 in stem aqueous extract (0.006 ± 0.00 mg/ml) and leaves water extract (0.007 ± 0.00 mg/ml) as compared to SAR pH 5.0 and 6.0 showered Guar plants. When comparing these results with the control as exhibited (Fig. 5), the reducing sugar contents were found higher than all SAR treated Guar plant.

According to results exhibited (Fig. 6) the different parts of 20% ethanol extracts of *Cyamopsis tetragonoloba* L. (Stem, leaves, fruit) were quantified to check the effect of SAR of different pH with nitric acid on the concentration of plant-reducing sugar. It was observed that the reducing sugar contents were also low as compared to control plants which were not treated with SAR. However, when the amount of reducing sugar from SAR of different pH of nitric acid sprayed plants compared to each other. The resulting of this comparison the maximum concentration of reducing sugar was examined in fruit ethanol extract of SAR pH 6.0 treated plants' segments (0.0125 ± 0.0004 mg/ml), leaves ethanol extract (0.0094 ± 0.0004 mg/ml), and stem ethanol extract (0.007 ± 0.0006 mg/ml), while the minimum concentration of reducing sugar was measured in stem ethanol extract of pH 5.0 of SAR showered under study plants (0.005 ± 0.0004 mg/ml), leaves extract (0.0082 ± 0.0004 mg/ml). However, these results are comparatively lower than control grown plants (without any addition of pH solution of nitric acid used as SAR) as well as also aqueous extracts of SAR treated plants' segment.

Quantification of total protein from 20% aqueous and ethanol extracts of SAR treated Guar plants: Different extracts of *Cyamopsis tetragonoloba* L.'s segments were used to analyze for quantification of total protein. The result indicates that the highest range of total protein was investigated in fruit aqueous extract at pH 6.0 of SAR treated Guar plants (2.06 ± 0.10 mg/ml), meanwhile total protein contents were also estimated from other parts of under study plants and found better results than other simulated acid rain treated plants' segments as leaves aqueous extract (0.72 ± 0.030 mg/ml) and stem extract (0.532 ± 0.009 mg/ml) as compared to other SAR sprayed Guar plants. However, these results found low when compared with control (without spraying of SAR). Furthermore, the minimum quantity of total protein was noted in stem aqueous extract of pH 5.0 and 4.0 of SAR applied to different parts of plants as presented (Fig. 7) these results are also lower than pH 6.0 of SAR applied plants.

According to the result of total protein estimated from the ethanol extracts of 20% different parts of *Cyamopsis tetragonoloba* L. as illustrated (Fig. 8). A similar trend was noted as found in aqueous extracts of SAR applied under-investigated different parts of Guar plants where high contents of proteins were detected at pH 6.0 of SAR-added plants' segments. However, these results are low as compared to the control but higher than the other pH of SAR-incorporated Guar plants. The maximum range was considered as usually obtained in fruit ethanol extract at pH 6.0 (1.95 ± 0.010 mg/ml), leaves ethanol extract (0.66 ± 0.016 mg/ml) and stem ethanol extract (0.45 ± 0.020 mg/ml). While at pH 4.0 minimum range of total protein was calculated in stem ethanol extract (0.306 ± 0.059 mg/ml) and leaves ethanol extract (0.464 ± 0.011 mg/ml). In control, total proteins were calculated higher rather than SAR treated plants.

Quantification of total antioxidant activity from 20% aqueous and ethanol extracts of SAR treated Guar plants: Phytochemicals are important for plants and provide them defense against harmful insects and plant pathogenic microbes. These phytochemicals are also beneficial for human beings and have been used since ancient times as folk medicine to cure different infectious diseases. In the present study, some phytochemicals were analyzed quantitatively and qualitatively from different pH SAR-showered Guar plants' segments.

The results of antioxidant activity from 20% aqueous extracts of different segments of *Cyamopsis tetragonoloba* L. as illustrated (Fig. 9) were uppermost in fruit aqueous extract at pH 6.0 (0.057 ± 0.0016 mg/ml), leaves aqueous extract (0.041 ± 0.001 mg/ml) correspondingly. However, the lowest quantity of antioxidants was found at pH 4.0 in stem aqueous extract (0.035 ± 0.002 mg/ml), and leaves extract (0.036 ± 0.001 mg/ml) as compared to pH 6.0 and 5.0 respectively. Whenever these results were contrasted with the control, were seen as slightly higher than SAR treated Guar plant.

The antioxidant activity was observed from different 20% extracts of *Cyamopsis tetragonoloba* L. results indicated that the maximum quantity of antioxidants was found in fruit ethanol extract at pH 6.0 (0.056 ± 0.0012 mg/ml), leaves ethanol extract (0.039 ± 0.0019 mg/ml), while minimum quantity of antioxidant was noticed at pH 5.0 (0.036 ± 0.001 mg/ml) in stem ethanol extract and in leaves ethanol extract (0.037 ± 0.0006 mg/ml). When contrasting these results with control as shown (Fig. 10) the antioxidant activity was found higher in control than all treated Guar plants.

Quantification of phenolic compounds from 20% aqueous and ethanol extracts of SAR treated Guar plants: The total phenolic compounds from different fragments of 20% aqueous and ethanol extracts of *Cyamopsis tetragonoloba* L. plant were also carried out and the results of soluble phenol compound were significantly found as higher as reported results of other parameters earlier in present study at pH 6.0 of SAR treated plants but lower than control (without addition of SAR solution) as depicted (Figs. 11-12). In addition, the highest amount of total phenol was calculated in fruit aqueous extract at pH 6.0 (0.660 ± 0.020 mg/ml), leaves aqueous extract (0.427 ± 0.016 mg/ml) and stem aqueous extract (0.35 ± 0.016 mg/ml) and lowest range of phenolic compound was observed in stem aqueous at pH 4.0 (0.185 ± 0.008 mg/ml), leaves extract (0.30 ± 0.016 mg/ml) as presented (Fig. 11).

According to results present (Fig. 12) the total phenolics were also analyzed from 20% extracts of *Cyamopsis tetragonoloba* L., the good quantity of phenolic compounds measured at pH 6.0 of SAR sprayed plants' segments of ethanol extracts as in fruit 0.601 ± 0.020 mg/ml, leaves 0.395 ± 0.020 mg/ml and stem (0.33 ± 0.025 mg/ml) respectively but these results are slightly lower than control. Whilst, the minimum quantity of phenol was detected in stem ethanol extract at pH 5.0 and 4.0 of SAR showered plants' segments. However, pH 5.0 and 4.0 solutions of HNO_3 incorporated plants used as SAR; fruits did not appear throughout the study.

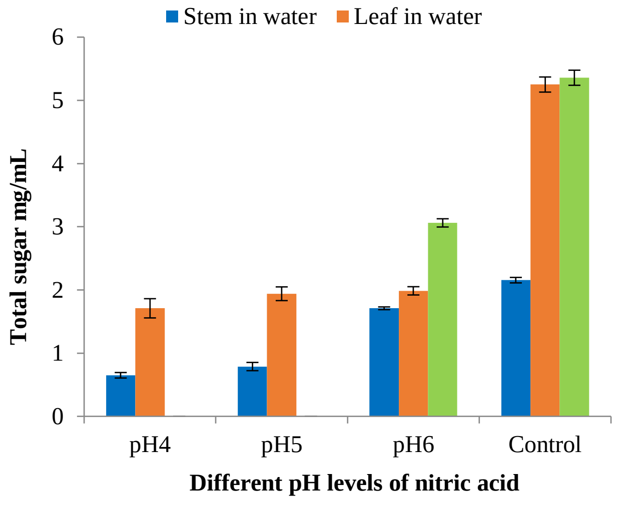

Fig. 3. Impacts of simulated acid rain (SAR) of different pH levels (HNO_3) on total Sugar of 20% Aqueous extracts of different segments of Guar Plants.

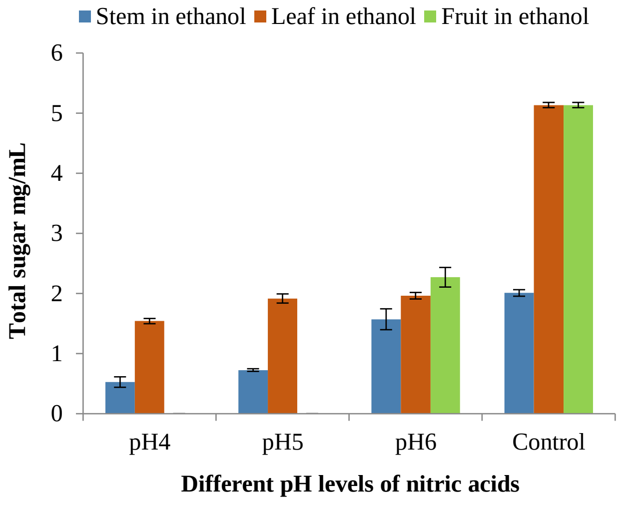

Fig. 4. Impacts of simulated acid rain (SAR) of different pH levels (HNO_3) on total Sugar of 20% Ethanol extracts of different segments of Guar Plants.

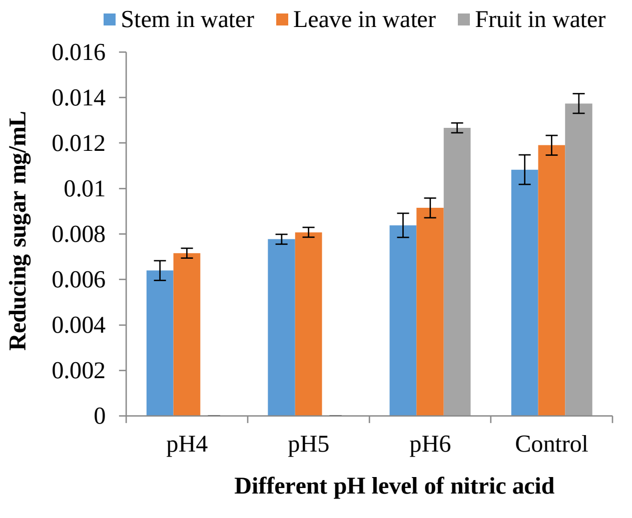

Fig. 5. Impacts of simulated acid rain (SAR) of different pH levels (HNO_3) on Reducing Sugar of 20% Aqueous extracts of different segments of Guar Plants.

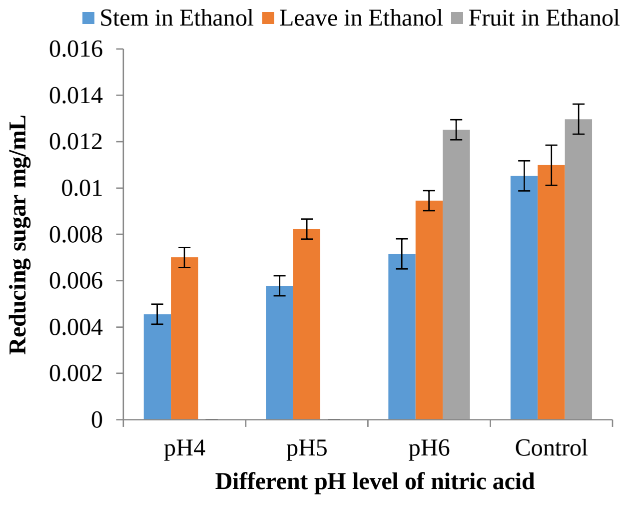

Fig. 6. Impacts of simulated acid rain (SAR) of different pH levels (HNO_3) on Reducing Sugar of 20% Ethanol extracts of different segments of Guar Plants.

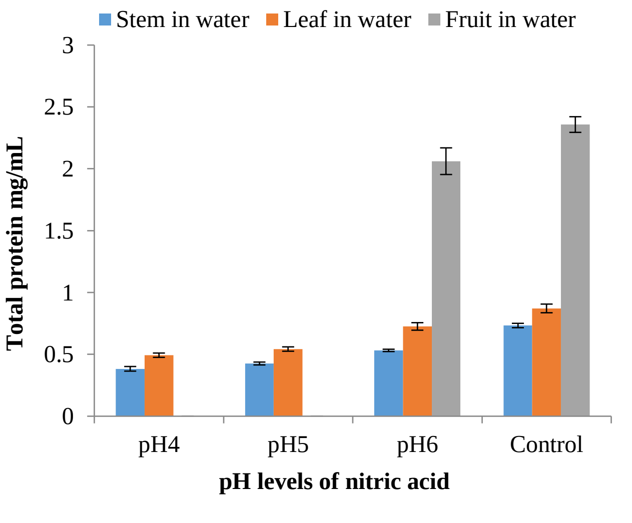

Fig. 7. Impacts of Simulated Acid Rain (SAR) of different pH levels of HNO_3 on the total protein of 20% aqueous extracts of different segments of Guar plants.

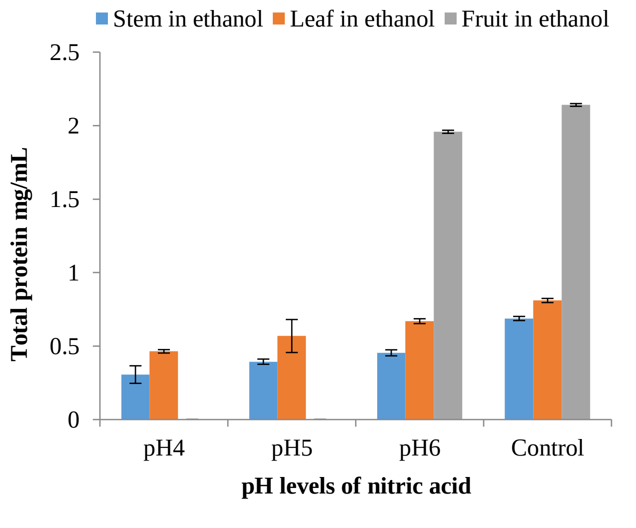

Fig. 8. Impacts of Simulated Acid Rain (SAR) of different pH levels of HNO_3 on the total protein of 20% Ethanol extracts of different segments of Guar plants.

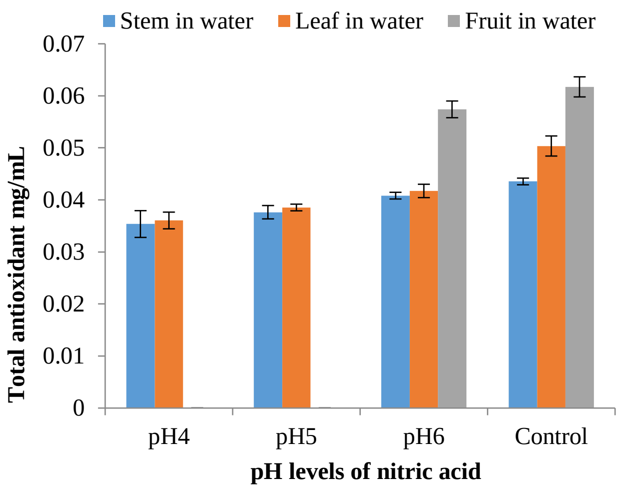

Fig. 9. Impacts of Simulated Acid Rain (SAR) of different pH levels of HNO_3 on the total antioxidant of 20% aqueous extracts of different segments of Guar plants.

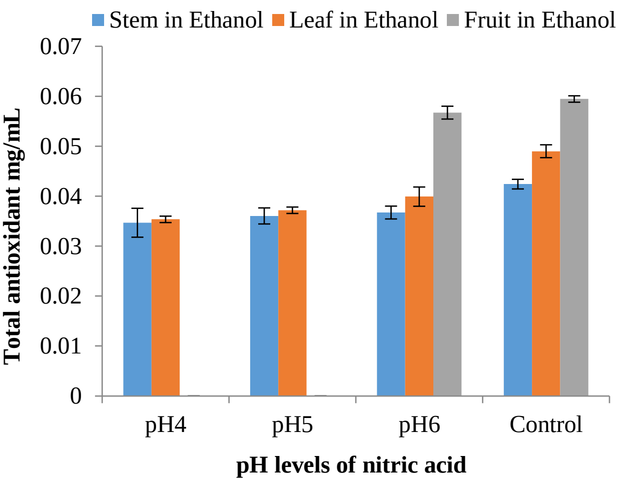

Fig. 10. Impacts of Simulated Acid Rain (SAR) of different pH levels of HNO_3 on the total Antioxidant of 20% Ethanol extracts of different segments of Guar plants.

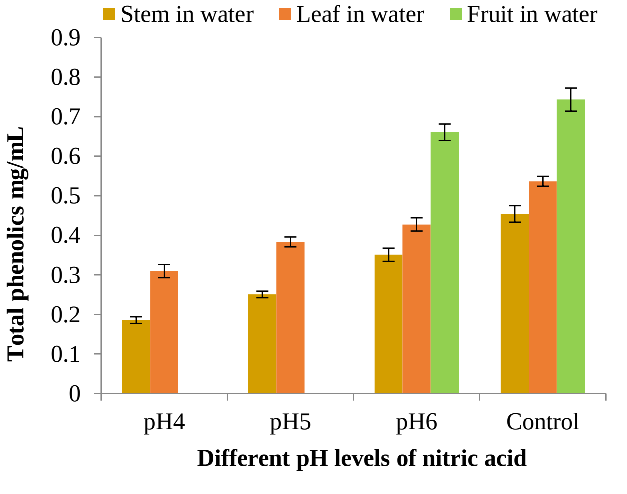

Fig. 11. Impacts of Simulated Acid Rain (SAR) OF different pH levels of HNO_3 on the Total Phenolic compounds of 20% aqueous extracts of different segments of Guar Plants.

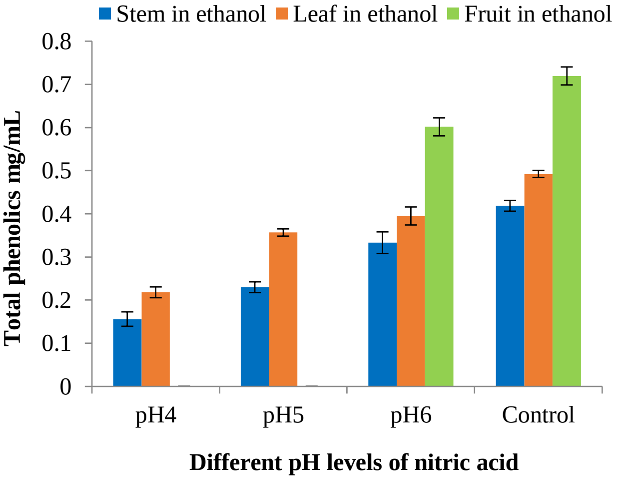

Fig. 12. Impacts of simulated acid rain (SAR) of different pH levels of HNO_3 on Total Phenolic compounds of 20% ethanol extracts of different segments of Guar Plants.

Qualitative analysis of alkaloids, flavonoids and tannins from 20% aqueous and ethanol extracts of SAR treated Guar plants' segments: The alkaloids were determined through the reported method. After the addition of chemicals in the stem, leaves, and fruit extract of *Cyamopsis tetragonoloba* L., reddish brown color precipitates were observed, indicated the presence of alkaloids. According to the results, the higher concentration of alkaloids was observed in the leaves extracts prepared in ethanol and aqueous extracts of fruit, the results were denoted by a plus sign (++++), whereas the lowest quantity was observed in the stem extract prepared in ethanol. Under the treatment of different pH of nitric acid used as SAR Guar plants, the highest amount of alkaloids was observed in aqueous extracts of leaves and fruit in control, while lowest concentrations were obtained in pH 4.0 in stem aqueous extract as exhibited (Table 3). The results were denoted by following signs: Low (+), moderate (++), high (+++) and highest (++++).

The flavonoids were examined qualitatively through color reaction reported method. When the reported quantity of chemical reagents was added to the 20% extracts of different parts (stem, leaves and fruit) of *Cyamopsis tetragonoloba* L., yellow precipitates were appeared, which indicated the presence of flavonoids. According to the results as shown (Table 4) the high (+++) contents of flavonoids were recorded in all plant extracts of Guar plants' fragments prepared in water at a pH of 6.0 SAR treated plants and the highest (++++) in control. Whereas the lowest quantity was found in the stem extracts prepared with ethanol and the other extracts solution was observed moderate density of flavonoids.

The presence of tannins was also checked from the extracts of each part of *Cyamopsis tetragonoloba* L. plants which were sprayed with various pH of nitric acid used as SAR. The greenish colored was observed when chemical reagents were incorporated in test solution, which indicated the occurrence of tannins in the test sample of Guar plants' extracts. The results are exhibited (Table 5), the highest (++++) and high (+++) tannins were found in leaves and fruit aqueous extract at pH 6.0 and control plants' segments extracts respectively. In addition, moderate results of tannin were examined in the plant extracts prepared in the ethanol. Whilst the least quantity was noted in stem and leaves ethanol extract of pH 4.0 SAR treated plants. Furthermore, it was also noted that no results were obtained from stem ethanol extracts of pH 4.0 and 5.0 SAR showered plants throughout study of alkaloids, flavonoids and tannins.

Table 3. Qualitative analysis of alkaloids from 20% extracts of different segments of Guar.

Treatments of different pH levels of HNO_3	Parts of plant	Extracts	
		Ethanol	Water
pH 4	Stem	-	+
	Leaves	+	++
pH 5	Stem	-	++
	Leaves	++	+++
pH 6	Stem	+	++
	Leaves	+++	+++
	Fruit	+++	+++
Control	Stem	++	+++
	Leaves	+++	++++
	Fruit	+++	++++

Table 4. Qualitative analysis of flavonoids from 20% extracts of different segments of Guar.

Treatment of different pH levels of HNO_3	Parts of plant	Extracts	
		Ethanol	Water
pH 4	Stem	-	+
	Leaves	+	++
pH 5	Stem	-	++
	Leaves	++	+++
pH 6	Stem	++	++
	Leaves	+++	+++
	Fruit	+++	+++
Control	Stem	+++	++++
	Leaves	+++	++++
	Fruit	+++	++++

Table 5. Qualitative analysis of tannins from 20% extracts of different segments of Guar.

Treatments of different pH levels of HNO_3	Parts of plant	Extracts through using solvents	
		Ethanol	Water
pH 4	Stem	-	+
	Leaves	+	++
pH 5	Stem	-	+
	Leaves	++	+++
pH 6	Stem	++	++
	Leaves	+++	+++
	Fruit	+++	++++
Control	Stem	++	+++
	Leaves	+++	++++
	Fruit	+++	++++

Discussion

Nowadays, in developed and underdeveloped countries urbanization, and industrialization are escalating day by day which is causing high pollution in the environment which may cause acidic precipitation in the environment and be harmful to nature. The consequence impacts of SAR were reported by several workers on various morphological parameters and the phytochemicals of various plants. However, it needs more work to explore the effect of simulated acid rain on different plants.

The adverse acid rain effects observed are mainly reliant on pH of the water. The acid rain of pH 3.0 or below may damage the segments of plants.(Liu *et al*., 2018a) have also stated that low-acidic rain may promote seed germination, improve the growth of plant tissue, and enhance the accumulation of biomass in plants.

Acid rain influence as an abiotic factor that affects various metabolic processes and physiological developments of plants and is also involved in the prominent reduction in the normal growth and development of plants (Raoufi *et al*., 2023).

It was reported (Raven *et al*., 2005) that germination relies not only on external factors but also on internal conditions within the seed such as water, light or shadow, oxygen, and temperature. The existing results revealed that the Guar seeds exposed to simulated acid rain at pH 2.0, stopped the germination. However, at pH 3.0, 4.0, 5.0, and 6.0 applied seeds, the germination rate rose with increasing pH as compared to control. Similar findings were also documented by (Pham *et al*., 2021) studied on influence of SAR on the growth and yield of soybeans. It was reported that the germination rate decreased significantly with rising acidity levels. The rate of germination recorded 78% at pH 3.0 and 83% at pH 3.5 were lower than low acidic pH. These results confirm our findings that which Guar germination rate ascended 54% at pH 3.0 and 66% at pH 4.0 and also increased as the range of pH increased.

On the other hand, (Zabawi *et al*., 2008) and (Kohno, 2017) also revealed the effects of SAR with various ranges of pH on germination of Rice seeds and stated that the rate of germination was significantly lessened at pH 3.5, as compared to the other SAR pH levels and there was also no any remarkable outcome on germination between the range of pH 4.0, 4.5 and control (pH 5.6) of SAR. These studies of SAR were also in harmony with (Rabani & Ezati, 2015) on germination of wheat and reduction recorded 60 and 50% at pH 3.0 and 2.5 correspondingly.

The present results show the effects and deficiency symptoms in Guar plant maturation absent fruit and flower, and length of the shoot was much diminished, and the branch did not appear at pH 3.0, 4.0, and 5.0. The results of this study showed that as simulated acid rain pH decreased (6,5,4,3), the leaves' length also decreased. Previous studies reported similar findings on simulated acid rain but in other plant species. (Lal and Singh 2012) have investigated the influence of SAR on sunflower plants which exhibited lessening in length of leaves (9.36 cm) at pH 5.7. Furthermore, the size of the sunflower leaves was also more affected and reduced at pH 4.5 and pH 3.0 (8.50 and 6.65 cm) respectively.

The different pH (2.5, 3.5 and 5.5) of SAR also badly affected the size of wheat leaves (15.3 cm, 21.9 cm and 25.6 cm) (Rabani & Ezati, 2015). However, (Chen *et al*., 2023) observed slightly dissimilar inclinations in *Mirabilis jalapa* Linn., as the pH level of simulated acid rain declined, the size of leaves first ascended and then decreased.

The leaves were in direct interaction with simulated acid rain. Therefore, signs of acid rain on Guar plants were clearly shown on the leaves' surface. Leaves changes were observed during simulated acid rain treatment, and pathological phenomena appeared on the leaves. At the more intense (pH 3.0 and 4.0) treatments, discolored leaves and curled leaves edges appeared on the Guar leaves surface and some of the leaves were punctured with small holes. In particular, at (pH 3.0), the leaves were necrotic. Visual observation of the color of the leaves showed that the lower the pH, the more the green color of the leaves faded.

Simulated acid rain influences the growth of leaves by necessary nutrient absorption and evapotranspiration, moisture in leaves, leaves characteristics and environmental factors (Dickison, 2000). The present findings also agreed with other investigations. (Liang & Zhang, 2018) revealed that simulated acid rain of pH 5.6 highly affected the morphological characters of *Asparagus cochinchinensis.* When the pH of acid rain increased especially at pH < 4.0, the leaves of the plant shaded, similar color, damaged, curling and wilted. Alike, the results examined by (Imran *et al*., 2014) that at pH 3.5 of nitric acid or sulphuric acid badly pretentious to all morphological characters containing shoot length, water contents of the shoot, number of leaves, root ratio, and potassium ion concentration.

According to Pham *et al*., (2021), When SAR of pH 3.0, 3.5, and 4.0 were applied to soybean plants, the leaves of the plants exhibited shorter and damaged as compared to strongly acidic pH-treated plants. (Zhang *et al*., 2020) have reported that a low acidic pH 3.5 or 2.5 of SAR usages on *Camellia sinensis* plant adversely damaged the leaves and also badly influenced root length and plant height. (Ramlall *et al*., 2015) have investigated the effects of simulated acid rain on *Trichilia dregeana* seeds. They found that pH 3.0 and 4.0 tested seedlings showed signs of stress directly linked with acid rain: leaves tip necrosis, irregular bi-lobed leaves tips, necrotic leaves spots, and chlorosis. (Odiyi & Eniola, 2015) have studied the simulated acid rain effect on the Cassava plant and found that SAR low pH (2.0 and 3.0) caused serious chlorosis, necrotic lesions and leaves tip injuries as compared to pH 5.

The impact of SAR was seen significantly on various phytochemical parameters of the Guar plant in present study. These research crop parameters were evaluated and then values were documented. The noteworthy impacts of SAR were also noted in the present study on total sugar and reducing sugar and other phytochemicals of Guar plant.

The analysis showed that the maximum quantity of total sugar and reducing sugar was found at pH 6.0 and the minimum quantity of total sugar and reducing sugar was found at low pH such as pH 3.0 and 4.0. However, control without incorporated SAR of different pH-grown plants' phytochemicals was recorded higher than SAR-treated Guar plants throughout present study. The impact of SAR on rice, wheat, mustard and tomato (*Lycoperiscon esculentum* Mill.) at low pH 4.0 and 5.0, the reducing sugar reduced to a greater degree than total sugar (Zeng *et al*., 2005; Shaukat & Khan, 2008). Similarly, (Zhang *et al*., 2023) have argued that the total sugar content of the three SAR-treated plant species (*Pinus massoniana, Phyllostachys edulis and Phyllostachys edulis*) reduced with a decrease in the pH level. (Debnath *et al*., 2018c) have also revealed the same findings of acid rain stress on tomatoes' sugar contents. (Li, 2009), stated SAR stress on sugar aggregation in tomato plants and found falling of total carbohydrates contents in leaves at strong pH treatment as reported in existing study.

(Bao *et al*., 2019) revealed that decreasing the concentration of plant sugars in strong acidic rain caused the cell membranes damaged, enhanced permeability and improved plant seedlings' requirement for reducing sugar synthesis, energy and nutrients. The low pH of acidic SAR causes the growth, carotenoids, chlorophyll, soluble sugar and protein contents of tomato leaves of tomato. The accumulation of enzymatic antioxidants in tomato plants at pH 3.5 and pH 2.5 of SAR, caused the declination of enzymatic oxidants (Debnath *et al*., 2018a).

The Guar plant is rich in protein content. Simulated acid rain adversely affected different pH levels on Guar plant as compared to control. The present study indicates that protein content is drastically reduced at low pH 4.0 and 5.0 as compared to control and pH 6.0. (Zhang *et al*., 2023) have documented the effects of SAR on protein contents of three plants *Pinus massoniana*, *Phyllostachys edulis*, and *Cinnamomum camphora*. The higher concentration of protein in these plants was observed at slightly acidic SAR as compared to control but the protein contents reduced at strong acidic rain. It is also reported (Bao *et al*., 2020) that the SAR decreases enzyme activity that produces plant proteins hence decreasing the protein contents. On the other hand, similar findings were also reported by (Chen *et al*., 2013) that as SAR pH decreased the protein content also decreased in *Liquidambar formosana* and *Schima superba* plants.

An antioxidant is a chemical that prevents unwanted oxidation, particularly one that is used to alleviate the disintegration of stored foods. The workers in this field reported that the non-enzymatic antioxidants play a pivotal role in maintaining the structure, function and redox status of the cell (Chen *et al*., 2010). Similarly, abiotic stresses, like AR emphasize in plants upsurge the synthesis of phenolic, proline and flavonoid, which provide assistance to indirect ROS scavenging, advancement of cellular signaling and intracellular redox-homeostasis rebuilding (Zhang *et al*., 2020). SAR influenced Guar plant's antioxidants at different pH levels in present investigation. The analytical present study of antioxidant activity of Guar stated that the maximum amount of antioxidants is observed in aqueous fruit extract at pH 6.0 rather than in fruit ethanol extract plant treated with SAR. (Kacharava *et al*., 2013) have stated that the high acidity stress may cause induce changes in the antioxidant metabolism system.

The results of SAR application indicate that in both aqueous and ethanolic extracts of leaves, stem and fruit of Guar plant as pH of SAR reduced (pH 6.0,5.0,4.0) the amount of phenolic also declined. A significant amount of phenols was found in present study at low pH indicated that phenol production in response to exposure to the function of these compounds in modifying plant defense mechanism under stress conditions. A similar observation was reported by (Kacharava *et al*., 2013) when a study on *Triticum timopheevi* plant. Nevertheless, it was revealed that the non-enzymatic activity involving phenolic, proline and flavonoids enhanced in tomato plantlets beneath AR stress (Debnath *et al*., 2018b). On the other hand, it was also stated that non-enzymatic and enzymatic activities were triggered by relentless AR stress in tomato plants. Debnath *et al*., (2018c) have also found that strong acidity (pH 2.5) of SAR causes lesser flavonoid contents than SAR of pH 3.5 usage. It was also revealed (Debnath *et al*., 2021) that total antioxidant potency can be associated with the accumulation of phenolics and flavonoids and their activities depending on pH of simulated acid rain and type of plant cultivars.

According to the above discussion, it can be abridged that under mild AR stress circumstances, non-enzymatic and enzymatic antioxidant activities may provide assistance in balancing ROS accretion and detoxification in plants nonetheless the severe AR stress conditions can destroy the capability of detoxification of plants. Furthermore, plants compensate for oxidative destruction by upregulating non-enzymatic and enzymatic antioxidant compounds under SAR stress but subsequently getting at higher AR stress, plant's antioxidant compounds accumulation again begin to decline due to failure of their defensive system and plants cannot reimburse ROS scavenging antioxidant compound under over stress by SAR.

Consequently, overall present study, strong pH simulated acid rain exhibited a negative impact and severely affected not only morphological characters as well as phytochemicals of Guar plants. The germination rate, the leaf area, the stem length, number of branches and the number of fruits decreased when the low pH of acidic rain showered.

Conclusion

The intent of the existing study is to investigate the effect of simulated acid rain on germination, morphology and phytochemicals of Guar. Based on observations and results obtained from present research, it is concluded that the germination and growth of Guar was significantly ceased by SAR of pH 2.0 and pH 3.0 of HNO_3 and negatively influenced the guar plant. Nevertheless, SAR at pH of HNO_3 (pH 4.0 and pH 5.0) showered plants exhibited some leaves and shoots but were badly influenced by SAR and caused chlorosis, wilting, early leaf senescence etc. Moreover, the number of leaves, shoot length, and number of fruit remarkably increased at pH 6.0. It can be deduced based on observations of their morphological characters, physiology, yield and phytochemicals that among at different pH ranges of SAR, Guar better resisted SAR treatment of pH 4.0, 5.0 and 6.0. However, under study guar plants were more resistant particularly at pH 6.0. Similar results were also observed during the analytical study of Guar's secondary metabolites like carbohydrates, total protein, total antioxidant and phenol reduced as the increase acidity of SAR. The least quantity was observed in pH 4.0 and 5.0, while maximum quantities were observed at pH 6.0 of SAR but lower than control. The present study revealed that the simulated strong acidic rain badly affected the under-study plant. In this regard to resolve the issues of acid rain, it is advocated that the policymakers should construct a mutual collaboration bridge with scientists and stakeholders to convince and bind them to reduce the emission of pollutant gases that cause environmental pollution and then become acid rain. They should also explore alternate pollution-free resources of energy, focus on the values, goals to protect harmful effects on ecosystems, agriculture infrastructure and human beings and also guide the farmers about the harmfulness of Acid Rain and its solutions. If acid rain falls due to environmental pollution, the soil becomes acidic and inappropriate for farming, the farmer should neutralize soil acidity by using chalk or slaked lime which can enhance the fertility of agricultural land and can also avoid declining in crop yield and harmfulness of livestock.

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