METHODOLOGY

(i) Phytochemical studies

The procedures followed in the present work for the extraction, isolation and identification of phytochemicals are described below.

Flavonoids

Five grams of leaf powder was extracted in a soxhlet with methanol for 48 hrs till the plant material became colourless. The methanolic extract was concentrated to dryness in a water bath. 25-30 ml of water was added to the dry residue and the water soluble phenolic glycosides were filtered out. The filtrate was hydrolysed in a water-bath for one hour using 7% HCl. This hydrolysate was extracted with diethylether/solvent ether, whereby the aglycones got separated into ether fraction (Fraction A). The remaining aqueous fraction was further hydrolysed for another 10 hr to ensure the complete hydrolysis of all the O-glycosides. Aglycones were once again extracted into diethyl ether (Fraction B) and the residual aqueous fraction was neutralized and evaporated for the analysis of glycoflavones.

Ether fractions A and B were combined and analysed for aglycones using standard procedures (Harborne, 1967, 1984; Mabry et al., 1970; Markham, 1982). The combined concentrated extract was banded on whatman No. 1 paper and chromatographed alongwith quercetin as the reference sample. The solvent system employed were Forestal (Con. HCl : Acetic acid : Water : 3:30:10) or 30% glacial acetic acid. The developed chromatograms were dried in air and the visibly coloured compounds were marked out. These

papers were observed in Ultraviolet light (360 nm) and the bands were noted. Duplicate chromatograms were then sprayed with 10% aqueous Na_2CO_3 and 1% $FeCl_3$ and the colour changes were recorded. R_q (R_f relative to quercetin) values were calculated for all the compounds. The bands of compounds were cut out from unsprayed chromatograms and were eluted with spectroscopic grade methanol. The UV absorption spectra of these compounds were recorded in methanol using 'Shimadzu UV 240' recorder type spectrophotometer. The bathochromic and hypsochromic shifts induced by the addition of various reagents were studied. The reagents used and their preparation are given below :

Sodium Methoxide (NaOMe) : Freshly cut sodium metal (2.5 gms) was added cautiously in small portions to dry spectroscopic methanol (100 ml). The solution was stored in a tightly closed glass bottle.

Aluminium Chloride ($AlCl_3$) : Five gms of fresh anhydrous AR grade $AlCl_3$ (which appeared yellow-green and reacted violently when mixed with water) were added cautiously to spectroscopic methanol (100 ml).

Hydrochloric acid (HCl) : Concentrated AR grade HCl (50 ml) was mixed with distilled water (100 ml) and the solution was stored in glass stoppered bottle.

Sodium acetate (NaOAc) : Anhydrous powdered AR grade NaOAc was used.

Boric acid (H_3BO_3) : Anhydrous powdered AR grade H_3BO_3 was used.

The concentrations of the sample solution prepared by eluting chromatogram strips were adjusted so that the optical density (OD) fell in the region of 0.6 to 0.8. The methanol spectrum was taken using 2-3 ml of this stock solution. The NaOMe spectrum was measured immediately after the addition of three drops of NaOMe stock solution to the flavonoidal solution used for methanol spectrum. The solution was then discarded. The $AlCl_3$ spectrum was measured immediately after the addition of six drops of $AlCl_3$ stock solution to 2-3 ml of fresh stock solution of the flavonoids. The $AlCl_3$/HCl spectrum was recorded next, after the addition of 3 drops of the HCl stock solution to the cuvette containing $AlCl_3$. The solution was then discarded. For NaOAc spectrum, excess coarsely powdered anhydrous AR grade NaOAc was added by shaking the cuvette containing 2-3 ml of fresh solution of the flavonoids, till about a 2 mm layer of NaOAc remained at the bottom of the cuvette. The spectrum was recorded 2 minutes after the addition of NaOAc. NaOAc/H_3BO_3 spectrum was taken after sufficient H_3BO_3 was added to give a saturated solution. The solution was discarded after recording the spectrum.

The structure of a flavonoid is established by its absorption maxima, shape of the curves, shifts (both bathochromic and hypsochromic) with different reagents and colour reactions. The identifications were confirmed by co-chromatography with authentic samples.

The aqueous fraction remaining after the separation of aglycones was neutralized by the addition of anhydrous Na_2CO_3/$BaCO_3$ and concentrated to dryness. When $BaCO_3$ was used

barium chloride got precipitated and was filtered out. This filtrate was concentrated to dryness. The alcoholic extract of the dried residue was banded on whatman No.1 paper and the chromatogram was developed with water as solvent system. Glycoflavones were visualized by their colour in UV and with 10% Na_2CO_3 spray. Further analysis and identification were done using spectroscopic methods as explained before.

Phenolic acids

Analysis of phenolic acids in the combined ether fraction (A and B) was carried out by two-dimensional ascending paper chromatography. Benzene : acetic acid : water (6:7:3, upper organic layer) in the first direction and sodium formate : formic acid : water (10:1:200) in the second direction, were used as irrigating solvents. The sprays used to locate the compounds on the chromatograms were diazotised *p*-nitraaniline or diazotised sulphanilic acid and a 10% Na_2CO_3 over spray (Ibrahim and Towers, 1960).

Diazotization : 0.7 gms of *p*-nitraaniline/sulphanilic acid was dissolved in 9 ml of HCl and the volume made upto 100 ml. Five ml of 1% $NaNO_2$ was taken in a volumetric flask and kept in ice till the temperature was below 4^oC. The diazotized sprays were prepared by adding 4 ml of *p*-nitraaniline/sulphanilic acid stock solution to the cooled $NaNO_2$ solution. The volume was made up to 100 ml with ice-cold water.

The various phenolic acids present in the extract were identified based on the specific colour reactions they produce with the spray reagents and the relative R_f values in different solvent systems.

Tannins

Tannins are extracted in water and are tested by treating them with protein solution. To the water extract prepared by boiling 5 gm plant material in about 50 ml water, 2% freshly prepared gelatin solution was added. The formation of a white (or milky) precipitate showed the presence of tannins in the plant material (Hungund and Pathak, 1971).

Saponins

About 5 gm of the powdered leaf material was boiled with 50 ml water for half an hour. This extract was filtered, the filtrate was taken in a test-tube after cooling and shaken vigorously (to froth) for a minute or two. The formation of a persistant froth of 1 cm length showed the presence of saponins (Hungund and Pathak, 1971). Foam formation takes place even during aqueous extraction if the concentration of the saponins are more in the plant materials (Harborne, 1984).

Proanthocyanidins

For testing the presence of proanthocyanidins, about 5 gms of finely chopped (fresh) leaf material / 2 gm dry powdered material was taken in 20 ml test-tube and covered with approximately 5 ml of 2N HCl. Extraction was carried out by

placing the test-tube in a boiling water bath for half an hour. The extract was decanted after cooling and shaken with amyl alcohol. Presence of a red or near-carmine colour in the upper alcohol layer denoted a positive reaction for proanthocyanidins. An olive-yellow colour represented a negative reaction (Gibbs, 1974).

(ii) Allelopathic studies

Certified grains of wheat (Triticum aestivum L.) var. Lok. 1 and paddy (Oryza sativa L.) var. GR 11 were procured from Gujarat Rajya Beej Nigam, Vadodara. The wheat variety and rice variety were selected from a number of varieties after subjecting them to trials of germination, susceptibility to fungal attack and sustainability.

The seeds of Cassia tora and Cassia occidentalis were collected from in and around M.S. University Campus.

Seeds selected for uniformity of size, shape and colour were washed with water, surface sterilized with ethyl alcohol and were washed thoroughly with water to remove even the traces of alcohol.

Preparation of the extract

For preparing the extract 5gm of dry powdered plant material was refluxed with 100ml water, in a round bottomed flask, fitted with water/air condensor, for 2 hours in a water bath. The extract thus obtained was cooled and filtered. The filtrate formed the stock solution.

Experimental details

The stock solution thus obtained formed 100%. This stock solution was diluted with distilled water to make 10%, 20%, 40% and 60% concentrations. Distilled water served as control. Two replicates were kept for each treatment and each treatment was repeated twice. The seeds obtained after rinsing were transferred to sterilized petridishes (9.0 cm diameter), each lined with single layer of filter paper, moistened with adequate amount (10 ml) of distilled water in case of control and respective amount of extracts in treatments. Each petridish contained 20 seeds. The seeds were set for germination at 30 $\pm$ 2^{o}C under laboratory conditions. The parameters looked for were germination percentage, root length, shoot length, and dry weight / biomass.

i) Seed germination : Germination was recorded as the emergence of (> 2 mm) radicle. The measurements were made at an interval of 7 days from the day of imposition of treatments. Germination percentage was expressed in relation to control.

ii) Root and shoot length : Root length and shoot length of normal healthy seedlings were noted. The observations were recorded after 7 days interval from the imposition day of the treatments. The root and shoot lengths were calculated by averaging the root and shoot lengths of all the plants of each treatment. The data were then subjected to statistical analysis. The same seedlings after recording the observations for root/shoot length were used for recording dry weight.

iii) Dry weight : Dry weight of the seedlings were obtained by drying the seedlings in an oven at 60°C followed by weighing the seedlings till constant weight, the weight thus obtained forms the dry weight of the seedlings. The weight was averaged and calculated per seedling weight.

Statistical Analysis

Data obtained for root length and shoot length were statistically analysed using two way classification model with 20 observations per cell with interaction effect. Using ANOVA, equality of treatment (average means) and equality of concentration (average means) was tested and found to be very significant. To establish the hierarchy, student's 't' test was applied. The results obtained are at 5% level of significance.