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Analysis of Phytochemical, Antioxidant and Anti-Inflammatory Activity of *Cissus quadralangularis*

Kavya G[1,2], Akshatha M. D[1] and Selvam Arjunan[2]*

[1]NMKRV College for Women, Department of Biotechnology, Jayanagar, Bangalore - 560011, India.

[2]Department of Biotechnology, Faculty of Life & Allied Health Sciences, Ramaiah University of Applied Sciences, University House, Gnanagangothri Campus, New BEL Road, MSR Nagar, Bangalore-560 054

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Corresponding Author Email: biosel2007@gmail.com

Abstract

Cissus quadrangularis, a climber belonging to vitaceae family, is widely used in ayurvedic medical practices and believed to promote bone fracture healing. It is also used for the management of obesity, diabetics, asthma, menstrual discomfort, stomach ulcers, etc. Although traditional system of medicines values the plant for its profile and biological activity of plant for its medicinal properties, vigorous scientific analysis of the phytochemical studied are limited. This study looks at the phytochemical profile of the stem and node extract of *C. quadrangularis* and analyzed the antioxidant and anti-inflammatory of this extract to facilitate more controlled future studies to optimize a process for efficient *in vitro* propagation of the plant. Chloroform and Ethanol extracts from the plant were assayed qualitatively for secondary metabolites. The antioxidant activity of extracts was studied using DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) and electrochemical methods. Anti-inflammatory activity was analyzed using *in vitro* propagation method for standardizing C.quadrangularis by optimizing media composition and incubation conditions. The study showed that saponins, alkaloids, carbohydrates, cardiac glycosides and terpinoids are present in the plant extract of Cissus quadralangularis. Along with high concentration of phenolics, the extracts exhibited significant antioxidant and anti-inflammatory activity. Furthermore, media formulation that facilitated callus initiation from stem explants was developed.

Keywords

Cissus quadrangularis, Phytochemical analysis and Plant tissue culture.

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INTRODUCTION

Cissus quadrangularis L. is commonly known as "Hadjod" (In Hindi Had=bone ; Jod=to fix) is a climber plant species, which is endemic to India, Srilanka, Pakistan and other tropical countries belongs to the *vitaceae* family. This valuable medicinal plant is widely used in India. It is popular among the traditional healers having expertise in treating bone

related troubles. It is one of the very frequently used herbs by traditional bone setters of India. It is also used for treating piles, asthma, digestive troubles, cough, and loss of appetite. It can be cultivated in plains, coastal areas, jungles and wastelands up to 500m elevation. The whole plant including all parts such as stems, leaves, roots are documented to possess medicinal properties in ethnobotanical surveys conducted by ethnobotanists in traditional system of medicine (1).

Cissus qudrangularis L. (Hadjod) belongs to vitaceae family is an indigenous medicinal plant of India. The use of this plant by the common folk for promoting fracture healing process is an old practice. The stem of *Cissus quadrangularis L.*is also reputed in Ayurveda as alterative, anthelmintic, dyspeptic, digestive, tonic, analgesic in eye and ear diseases, in the treatment of irregular menstruation and asthma, and in complaints of the back and spine. Scientific studies have revealed the *Cissus* extract to possess cardiotonic and androgenic property (2).

The *Cissus quadrangularis Linn*, has been recognized as a rich source of carotenoids, triterpenoids and ascorbic acid and is proved to have potential for medical effects, including "Gastro protective activity" in conjugation with NSAID therapy and in "Lipid metabolism and oxidative stress". *The Cissus quadrangularis L.* plant contains high amount of vitamin C, Carotene A, anabolic steroidal substances and calcium. Stem contains two asymmetric tetra cyclic triterpenoids; *C. quadrangularis* is promoted as a weight loss agent. *C.quadralangularis* by itself or in formulations like Cylaris and CORE and in the combination with Irving is gabonensis reduced body weight (3). In addition to weight reduction, *C.quadralangularis* also reduced blood glucose levels and serum lipids. It also alleviated insulin resistance and scavenged free radicals.

Antioxidant and antimicrobial activity are also reported with *C.quadralangularis* consumption. *C.quadralangularis* shows gastro protective and hepatoprotective properties, as well as suppresses chronic ulcers (4). *C.quadralangularis* also demonstrates anti-inflammatory and analgesic properties. In rats, *C.quadralangularis* reduced edema of the ears and paws. In addition, *C.quadralangularis* also shows anti-tumor activity (5). In Ayurvedic medicine, the extract of *C.quadralangularis* is used to enhance bone fracture healings.

However, the phytochemical content and pharmacological actions of C.quadralangularis, having medicinal potential remain unidentified by rigorous scientific research. This study looks at the phytochemical profile of the stem and node extract of C.quadrangularis and analyzed the antioxidant and anti-inflammatory of this extract to facilitate more controlled future studies to optimize a process for efficient in vitro propagation of the plant (6).

MATERIALS AND METHODS

Chemicals required: - Major salts, Minor salts, Vitamins, Iron-EDTA, $CaCl_2.2H_2O$, Sucrose, Glutamine, 1% Ceterimide, 2% Bavistin, Ascorbic acid, 70% Alcohol, Hypochloride, Citric acid, Activated charcoal, Sodium hypochloride, Benzoquinone, PBS Buffer, Diclofenac, Ethanol, Chloroform, Gallic acid, 7.5% sodium carbonate, FC-reagent, DPPH,

Hormones required: - BAP, 2,4-D, Kinetin, TDZ, NAA, IAA, Cystien

METHODS

The MS medium is a classic basal medium used worldwide for plant tissue culture (7). It is also recognized that half the strength of MS medium is adequate for culturing of many plant species.

Table 1: Composition of Murashige and Skoog's medium (1962)

Major	
$(NH_4)NO_3$	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
Minor Elements	
$MnSO_4.4H_2O$	22.3

H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
$FeSO_4.4H_2O$*	27.8
Na_2EDTA*	37.3
Organic constituents:	
Myoinositol	100
Glycine	2.0
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Sucrose	20,000

The MS media was used for plant tissue culture. The media was solidified by adding agar (8g/L). Macronutrients and micronutrients measured along with vitamins. This basic media was altered with different hormones to check the callus initiation for *C.quadralangularis.*

STERLIZATION OF EXPLANTS

Method 1: Explants were collected from greenhouse and these were washed with a mild detergent followed by running tap water. These explants were dipped in 1% citramide and ascorbic acid for 10 minutes with continues shaking. Then these are washed with water and kept in 2% bavistin, pinch of ascorbic acid for 30 minutes. After sterilization of explants washed with detergents, taken to sterilized LAF for surface sterilization. Firstly, they were sterilized with 70% alcohol for 5minutes and washed with sterilized distilled water followed by sterilization with hypochloride for 10 minutes, washed with sterilized distilled water.

Method 2: Explants were collected from greenhouse and soaked in ascorbic acid, citric acid, activated charcoal before detergent wash for 1 hour which is followed by running tap water and detergent wash as previous method and then they were taken to sterilize LAF. Explants were sterilized with 70% alcohol for 5 minutes and washed with sterilized distilled water followed by sterilization with hypochloride for 10 minutes, washed with sterilized distilled water.

Variation in hormone concentration and incubation methods:

The hormones play vital role in the initiation of callus. The variation in hormones and Incubation methods has different effect on the plant.

a. Keeping the MS media as standard we varied the hormone concentration. In first media contains 2,4-D 1ml, TDZ 1ml, the second media contains
b. 2mg/ml2,4-D, 1mg/ml TDZ, 2mg/ml IAA, 0.5%activated charcoal, the third media contains
c. NAA 2.0, BAP 1.0, 0.5%activated charcoal, 100mg/L Ascorbic acid, 50mg/L Citric acid, and fourth media contains
d. NAA2.0, BAP 0.5, 0.5% activated charcoal, 100mg/L ascorbic acid, 50mg/L citric acid and fifth media contains
e. NAA2.0, 2,4-D 1.0, BAP 0.5, 0.5% Activated charcoal, 100mg/L ascorbic acid, 50mg/L Citric acid.

To prepare the extract for phytochemical analysis of *C.quadralangularis:*

Chloroform Extract: 1kg of node of *C.quadrangularis* is chopped into small pieces and placed it in hot air oven for 24 hours after they were dried completely and ground into fine powder. 30% of powder were

mixed with same amount of chloroform and kept in magnetic stirrer for 24 hours and filtered it with whatsman paper to obtain filtrate. This filtrate is kept in boiling water bath at 40° c, 12 -24 hours with constant stirring, crude extract was synthesized (8).

Ethanol Extract: 1kg of node of *C.quadrangularis* was chopped into small pieces and placed it in hot air oven for 24 hours after they were dried completely and ground into fine powder. 30% of powder were mixed with same amount of ethanol and kept in magnetic stirrer for 24 hours and filtered with whattsman paper to obtain filtrate. This filtrate is kept in boiling water bath at 40° c, 12 -24 hours with constant stirring, crude extract was synthesized (9).

Cold water Extraction (CWE) is the process whereby a substance is extracted from a mixture via cold water. It is a type of fractional crystallization. The process generally involves taking a mixture of substances, dissolving them in warm water and then rapidly cooling the mixture.

Carbohydrates Test:

Molisch's Test- 2ml extract, α-naphthalene solution, alcohol shake well (2min), add 1ml of conc H_2SO_4 alongside of test tube. Deep blue violet color junction indicates presence of carbohydrate.

Test for reducing sugar:

Fehling's A and B mixture of 1ml and boiled, 1ml extracts, heated for 10 min water bath and check for appearance of brick red color precipitate indicates presence of reducing sugar.

Bendict's Test : Equal volume (2ml) of Benedict's solution and extract were mixed in test tube, heated in water bath and check for changes in color to yellow indicates presence of reducing sugar.

Amino acid test:

Ninhydrin test- 2ml extract, 3drops of lead acetate solution, boiled on water bath for 10 min. The presence of purple color indicates presence of amino acid.

Test for Tannin:

Ferric chloride test: extract, 3ml of ferric chloride solution. The presence of black color indicates the presence of phenols and Tannins.

Lead acetate test: 3ml extract, 3ml of lead acetate occurrence of white precipitate indicates the presence of Tannins and phenols.

Test for glycosides:

Killer-Killani Test: - 2ml extract, 1ml glacial acetic acid, 3 drop ferric chloride, Conc H_2SO_4. The occurrence of bluish green in upper layer and disappearance of reddish brown color. This indicates the presence of glycosides.

Test for Saponins:

Foam test- 2g of extract, 20 ml water shaken vigoursly to observe persistent foam. This indicates the presence of Saponins.

Test for Flavonoids:

Shinoda test: 5 drops of conc H_2SO_4, extract, 0.5g of mg turnings. Appearance of pink color indicates the presence of flavinoids.

Test for triterpenoids:

Salkowaski test: 2ml extract, 5 drops of conc H_2SO_4 shaken, appearance of greenish blue color indicates the presence of triterpinoids.

Mayer's test: 3ml filtrate, 1ml Mayer's reagent (Potassium mercuric iodide) appearance of white precipitate indicates the presence of Alkaloids.

Wagner's test : 1ml of wagner's reagent (iodine in potassium iodide) appearance of reddish brown precipitate -in alkaloids

Hager's test: 3 ml of filtrate, 1ml of Hager's reagent (saturated Picric acid), appearance of yellow precipitate indicates the presence of alkaloids.

Test for total phenolics:

The phenolic content of plant extract was determined by using Folin-Ciocalteu reagent. Gallic acid was used as a reference standard for plotting calibration curve. A volume of 0.5ml of the plant extract (100µg/ml) was mixed with 2 ml of Folin-Ciocalteu reagent (diluted 1:10 with de-ionized water) and were neutralized with 4 ml of sodium carbonate solution. The reaction mixture was incubated at room temperature for 30 minutes with intermittent shaking for color development.

The absorbance of the resulting blue color was measured at 765nm using double beam UV-VIS spectrophotometer. The total phenolic contents were determined from the linear equation of a standard curve prepared with gallic acid. The content of total phenolic compounds expressed as mg/g gallic acid equivalent of dry extract.

ANTI- INFLAMMATORY STUDIES OF PLANT EXTRACT:

Electrochemical Method: All experiments were done in a conventional three electrode electrochemical cell with bare graphite as working electrode, saturated calomel electrode (SCE) as reference electrode and platinum wire as an auxiliary electrode. 10 ml of buffer solution was taken in an electrochemical cell to it sample 1 was added as a analyte and cyclic voltammeter was recorded at potential -1 to +1 at a scan rate of 100mV. To the same electrochemical cell 0.1M benzoquinone is added again cyclic voltammeter was recorded. The same procedure was repeated for sample and the readings were compared.

Anti-Inflammatory Activity: Diclofenac sodium, a teroidal anti-inflammatory drug was used as a standard drug. The reaction mixture consisting of 2ml of different concentrations of plant extract (100-500µg/ml) and standard diclofenac sodium (100 and 200 µg/ml) and 2.8 ml of phosphate buffered saline (pH-6.4) was mixed with 2ml of egg albumin (from fresh hen's egg) and incubated at 27±1 °C for 15 minutes. Denaturation was induced by keeping the reaction mixture at 70°C in a water bath for 10 minutes. After cooling, the absorbance was measured at 660nm. Each experiment was done in triplicate and the average was taken. The percentage inhibition of protein denaturation was calculated by using the formula (10).

COMPOSITION	INCUBATION	OBSERVATION
(a). MS media, 2,4-D 1ml ,TDZ 1ml	Dark	No callus formation and explants started browning which led to necrosis
	Dim light	
	Light	
	Fridge	
(b). MS media, 2mg/ml2,4-D, 1mg/mlTDZ, 2mg/mlIAA, 0.5%activated charcoal	Dark	No callus formation and explants started browning which led to necrosis
	Dim light	
	Light	
	Fridge	
(c). MS media, NAA 2.0, BAP 1.0, 0.5%activated charcoal, 100mg/L Ascorbic acid, 50mg/L Citric acid	Dark	Initiation of callus, no browning
	Dim light	Contamination
	Light	Browning and contamination
	Fridge	No browning and no initiation of callus
(d). MS media NAA2.0, BAP0.5, 0.5% activated charcoal, 100mg/L ascorbic acid , 50mg/L citric acid	Dark	No browning and no initiation of callus
	Dim light	Browning
	Light	Browning
	Fridge	No browning and no initiation of callus
(e). MS media, NAA2.0, 2,4-D1.0, BAP0.5, 0.5% Activated charcoal, 100mg/L ascorbic acid, 50mg/L Citric acid	Dark	No browning and no initiation of callus
	Dim light	Browning and contamination
	Light	Browning and contamination
	Fridge	No browning and no initiation of callus

% inhibition=$\frac{A_C - A_S}{A_C}$× 100 (*Where A_s-absorbance of test sample, A_c-absorbance of control).*

RESULTS AND DISSUSSION

Among the two methods of sterilization that was followed, in method -1, the explant started browning which led to necrosis and resulted in the death of the plant, while in method -2 there were no browning in the explants. Hence, method -2 might give a good result because anti-browning agents weren't added in method -1 and therefore, the explant started browning potentially due to the production of phenols.

Callus initiation was seen with node explant after three months (Fig 2), however, within week of

initiation, callus started turning brown leading to its necrosis. Composition 'c' 'd' and 'e' didn't show browning in explants (Fig 1, 3, 4) when they were kept in dark and in cold conditions (11). Therefore, the explants may be sensitive to light. Fig 3 and 4 shows callus initiation i.e. swelling of the cells but not complete initiation. Further investigation can be done by changing the media composition to obtain a complete callus. Moreover, it was observed that callus was initiated only in dark conditions.

Figure 1

Figure 2

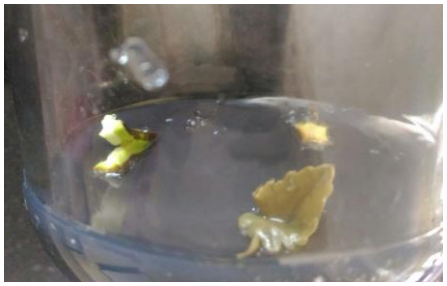


Figure 3

Figure 4

Figure 1: MS media, NAA2.0, BAP1.0, 0.5%activated charcoal, 100mg/L Ascorbic acid, 50mg/L Citric acid (No browning even after 3 months in dark condition).
Figure 2: MS media, NAA2.0, BAP0.5, 0.5% activated charcoal 100mg/L ascorbic acid, 50mg/L citric acid (Browning was seen within 2 weeks in light)
Figure 3 & 4: MS media, NAA2.0, BAP1.0, 0.5% activated charcoal 100mg/ acid, 50mg/L Citric acid (Callus was initialization).

As shown in the table 2 phytochemical analysis for *C.Quadrangularis* is that the tannins, flavonoids, saponins, alkaloids, carbohydrates, cardiac glycosides, terpenoids are present in plant extract of Cissus quadralangularis. Whereas the test for steroids and glycosides indicates its absence in the plant extract of Cissus quadralangularis.

Table 2: Results for phytochemical compounds

Phytochemical activity	Name of test	Plant stem extract
Tannins	FeCl test	+
Steriods	Salkowski test	-
Flavanoids	Shinoda test	+
Saponins	Frothing test	+
amino acid	Ninhydrin test	-
Alkaloids	Hager ,Meyer, and Wagner's test	+

Carbohydrates	Molisch test	+
Glycosides	Nitroprusside test	-
Cardiac glycosides	Keller killiani test	+
Terpenoids	Salkowski test	+

As shown in the table 3, the total phenolic content in the examined plant extracts using the Folin-Ciocalteu's reagent is in terms of Gallic acid equivalent. The value obtained for the concentration of total phenols are expressed as 300µg/ml Gallic acid of extract and this was taken as standard. The highest concentration of phenols was seen in ethanol extract when compared to chloroform extract.

Table 3 – Total phenolic content in the plant extracts expressed in terms of Gallic acid equivalent (µg of GA/ml of extract)

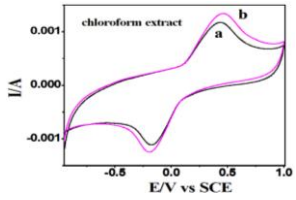
EXTRACT	µg of GA/ml of extract
Ethanol	2.144 ± 0.0181
Chloroform	0.160 ± 0.0155

Each value is the average of three analysis ± SD

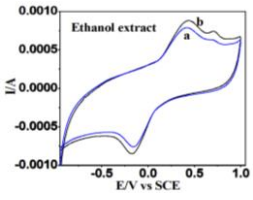
The total phenolic content in plant extracts of the *Cissus* species depends on the types of extract, i.e. the polarity of solvent used in extraction. High solubility of phenols in polar solvents provides high concentration of these compounds in the extracts obtained using polar solvents for the extraction. Kragujevac J. 2011 reported (12) that in ethanol extract the total phenolic content is 0.033±0.616 in leaves, however as seen in our studies the ethanol extract of nodes has much higher phenolic content (2.244± 0.0181). This may be because the plant node contains higher phenylalanine than leaves. Therefore, the phenolic content is high in stems than in the leaves.

Anti-oxidant activity and anti-Inflammatory activity studies of plant *C.quadralangularis:*

Cyclic voltammetry was recorded for the samples 1 and 2 and their results are compared to check their antioxidant activity. Antioxidant activity is said to be high if the sample is able to donate its electron easily. On the other hand, benzoquinone has an electron deficient site; hence it helps in detection of antioxidant activity. The amount of antioxidant capacity of a sample is detected by the area swept by the peak in cyclic voltammogram. Figure 5 represents peaks of plant extracted from chloroform, the peak current of the sample was 145mA (b) whereas after adding benzoquinone the peak current decreases to 133mA (a). The sample 2 was also tested, the peak current of plant extracted from ethanol was observed to be at 877µA (b) and after adding benzoquinone reduced to 786µA (a) represented in figure 6. Figure 7 represents peaks of sample 1 and sample 2, the area swept by the peak were recorded to be 440µC and 272µC respectively. This clearly implies that plant extracted from chloroform exhibits high antioxidant activity than the plant extracted from ethanol.



Figure



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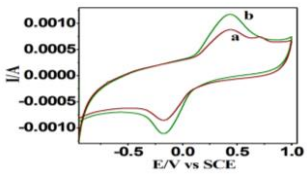

Figure 7

Figure 5, 6 and 7: Antioxidant Activity by Electrochemical Method

ANTI-INFLAMMATORY

The inhibitory effect of different concentration of *C.quadralangularis* extract on protein denaturation is shown in table 4. *C.quadralangularis* extract (25-100µg/ml) show significant inhibition of denaturation of egg albumin in a dose depend manner and the in-vitro anti-inflammatory activity of the extract was comparable to the diclofenac sodium, a reference drug (100-200µg/ml). A significant difference in the inhibition of thermally induced protein denaturation was observed with extract as compared with standard drug at concentration of 100µg/ml. However, at a concentration of 200µg/ml, inhibition activity of extract and diclofenac sodium was comparable.

Table 4- in-vitro anti- inflammatory activity

TREATMENT	CONC (µg/ml)	Inhibition of protein denaturation (%)
Stem extract	25	11.87±0.234
	50	18.75±0.223
	100	21.87±0.290
Diclofenac sodium	100	1.60
	200	1.64

Values are means ± SD

As the concentration of plant extract increased, the anti-inflammatory properties of *C.quadrangularis* increased when compared to reference drug Diclofenac sodium.

Anti-inflammatory activity was determined by using plethysmometer, one of the possible and frequently used animal models to screen anti-inflammatory drugs. The progress of carrageenan induced edema is bi-phasic, the first phase is release of histamine, serotonin and kinins and the second phase is the release of prostaglandins. Anti-inflammatory activity is due to flavonoids especially luteolin and by β-sitosterol. β-sitosterol present in methanol extract has ability to reduce the enzymes MPO indicating a reduction of neutrophils influx in the inflamed tissue. Calcium oxalate, carotene, tetraterpenoids, βsitosterol, amyrin and anabolic ketosteroids, are responsible for acceleration of healing and possess anti-inflammatory and analgesic activity. Ethanol extract exhibit protective effect on neutrophils mediated tissue injury induced by aspirin in rats. Methanol extract (90%) and dichloromethane extract of stems possess anti-inflammatory activity against COX. The stimulatory effect of extract is probably due to vitamins and is greater than that of the anabolic hormone durabolin (13).

CONCLUSION

Vitaceae family is valuable for the production of wine, medicine and perfumery. The plant *Cissus quandrangularis* used extensively *in alternate* medical systems to treat bone related diseases. Plant tissue culture of bone wine plant has not been standardized. This project was an effort to standardize the tissue culture condition for *Cissus quandrangularis.* One of the major problems encounter was browning or blackening of explants during callus induction. This was common phenomenon seen by other researchers as well. Callus induction of several plant species and exudation of phenolic substances is a natural mechanism in plants tissue culture. Leading to the browning of the medium and impairment of regeneration. Browning or necrosis can be avoided by using antibrowning agents like ascorbic acid (20mg/l) and charcoal which controls the oxidation of phenols. Due to the presence of tannins, flavonoids, saponins, alkaloids, carbohydrates, cardiac glycosides, terpenoid in plant extract of Cissus quadralangularis, the plant has more medicinal value like antibacterial, antifungal, antioxidant, and anthelmintic, antihemorrhoidal. It can be used for treating gastritis, bone fractures, skin infections, constipations, eye diseases, piles, anemia, asthma, irregular menstruation, burns and wounds. The leaves and young shoots are powerful alternatives. Powder is administered in treatment of hemorrhoids and certain bowl infections. The *C.quadralangulris* has anti-oxidant activity; hence, it has the capacity to accept electrons which is released by the body. These electrons may react with the cells which lead to many diseases like cancer. Therefore, it is a good anti-oxidant.

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APPENDIX

1. 2, 4 –D: 100mg of 2, 4 – D was weighed accurately and dissolved in a small volume of 95% ethyl alcohol or in KOH and then made up to volume 50 ml with double distilled water stored at 4^{o}C
2. NAA: 50 mg of NAA was dissolved in a small amount of in NaOH or KOH and made upto 50 ml with distilled water, stored at 4^{o}C
3. IAA: 50 mg of IAA was weighed accurately and dissolved in a small volume of 95% ethyl alcohol or in KOH and then brought to volume of 50 ml with double distilled water and stored in bottles or bottles covered with a black paper and kept in the dark since it is unstable in light.
4. Kinetin stock: 50 mg of Kinetin was accurately weighed and dissolved in NaOH and made up to the volume 50ml with distilled water and stored in amber bottles or bottles covered with a black paper and kept in the dark since it is unstable in light at 4^{o}C
5. BAP: 100mg of BAP was weighed accurately and dissolved in a small volume of NaOH and then made up to volume 50 ml with double distilled water stored at 4^{o}C
6. GA_3: Weigh 100 mg of Gibberlic and Add few drops of ethanol to dissolve GA_3make up the volume to 100ml