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‘’TO EVALUATE ANTI-OXIDANT ACTIVITY OF SOME SELECTED ANTI-CANCER PLANTS AND ITS HERBAL FORMULATION’’

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Abstract: This study aims to evaluate the anti-oxidant activity of some selected anti-cancer plants and their herbal formulation. Anti-oxidants play an essential role in scavenging free radicals and preventing oxidative damage, which has been implicated in the development of various diseases, including cancer. The selected plants and their formulation will be tested using various anti-oxidant assays to determine their total anti-oxidant capacity, radical scavenging activity, and reducing power. The results of this study could provide valuable insights into the potential of these plants and their formulation as source of natural anti-oxidants with anti-cancer properties.

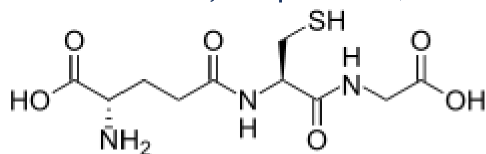
Key words: H_2O_2, NO_2, DPPH, ABTS, ELISA, 96 well plate, Brassica Nigra, Mentha Piperita, Glycyrrhiza Glabra, Cyperus Rotundus, anti-oxidant, anti-cancer.

Introduction: A substance which protects the cells from the damage caused by the free radicals which are unstable molecules made by the process of oxidation during normal metabolism. Free radicals may play a part in cancer, heart disease, stroke, and other diseases of aging.

Your body can also be exposed to free radicals from a variety of environmental sources, such as cigarette smoke, air pollution, and sunlight. Free radicals can cause “oxidative stress,” a process that can trigger cell damage. Oxidative stress is thought to play a role in a variety of diseases including cancer, cardiovascular diseases, diabetes, Alzheimer’s disease, Parkinson’s disease, and eye diseases such as cataracts and age-related macular degeneration.

Antioxidants are man-made or natural substances that may prevent or delay some types of cell damage. Diets high in vegetables and fruits, which are good sources of antioxidants, have been found to be healthy; however, research has not shown antioxidant supplements to be beneficial in preventing diseases. Examples of antioxidants include vitamins C and E, selenium, and carotenoids, such as beta-carotene, lycopene, lutein, and zeaxanthin.

Structure of anti-oxidant compound- **glutathione**.



Pro-oxidant activities:

Antioxidants that are reducing agents can also act as pro-oxidants. For example, vitamin C has antioxidant activity when it reduces oxidizing substances such as hydrogen peroxide; however, it will also reduce metal ions that generate free radicals through the Fenton reaction.

2 Fe^{3+} + Ascorbate → 2 Fe^{2+} + Dehydroascorbate

2 Fe^{2+} + 2 H_2O_2 → 2 Fe^{3+} + 2 OH· + 2 OH^-

The relative importance of the antioxidant and pro-oxidant activities of antioxidants is an area of current research, but vitamin C, which exerts its effects as a vitamin by oxidizing polypeptides, appears to have a mostly antioxidant action in the human body.

Need of the Study: This research paper was chosen because, we feel that nowadays and even back in time herbal medicines were used by people because of natural herbs used and because of the reduced side effects of the herbal medicine.

Anti-oxidant substances or medicines can be useful in a plethora of ways. Let's try to understand this, during normal metabolism process, oxidation occurs and it releases free radicals which cause cell damage. Now because of this cell damage, there are certain diseases which put human health in danger such as, diabetes, cancer, obesity and many more.

Now to reduce the risk of certain diseases there should be anti-oxidant intake which can reduce the oxidative stress and reduce the cell damage caused by the free radicals, which can improve the human health at last making human life healthier and happier.

Furthermore, in order to intake such anti-oxidant medicines, making an herbal formulation such as tablet will be the best option. The four extracts which are used in this formulation also possess anticancer activities which can also prevent causing cancer. All the four plants were extracted using water as solvent.

Data and sources of the data: For this study secondary data has been collected from different articles and research paper. The references are given at the end of the paper.

Theoretical framework: Firstly, all the resources were collected and gathered. After that the plan of work was prepared with first preparing the aqueous extracts of four different plants. Then after extraction the product was preserved for the anti-oxidant assays to be performed for the evaluation of anti-oxidant activity. The results were noted of the observation. Furthermore, herbal formulation was framed as per the results of the anti-oxidant evaluation and as such herbal tablets were formulated.

Plant Introduction:

1. ***Brassica Nigra*:**

- ***Biological source***: temperate regions of Europe and parts of Asia.
- ***Synonym*:** Indian mustard seed

Figure 1.1

- ***Family*:** Brassicaceae
- ***Chemical constituents**:* polyphenols, phenolic acids, flavonoids, carotenoids (lutein, beta carotene), alkaloids, tannins, saponins, terpenoids, glycosides.
- ***Uses**:* In addition, mustard may possess a plethora of pharmacological activities including anti-oxidants, anti-inflammation, and bacteriostatic and antiviral activity. Mustard has also been used to combat several illness such as cancer, obesity, depression, diabetes, and cataracts. It may be used for neurodegenerative disorders like Alzheimer's disease and Parkinson's disease.
- ***Plant part used for extraction**:* plant seeds.

2. ***Mentha Piperita*:**

- ***Biological source***: obtained from the fresh leaves of peppermint by steam distillation.
- ***Synonyms*:** Peppermint

Figure 1.2

- ***Family**: Lamiaceae*
- ***Chemical constituents***: cineol, limonene, menthofuran, menthol, and menthone, eucalyptol, and carophyllene.
- ***Uses***: It has a variety of therapeutic properties and is used in aromatherapy, bath preparations, mouthwashes, and topical preparations. It also has been a classic choice for treatment of nausea. It is also used to treat fever, cold, digestive, antiviral, antifungal and for throat and oral mucosa inflammation.
- ***Plant part used for extraction***: fresh leaves of plant.

3. *Glycyrrhiza Glabra*:

- ***Biological source***: Also known as licorice and sweet wood, is native to Mediterranean and certain areas of Asia.
- ***Synonyms***: Licorice, Jethimadh

Figure 1.3

- ***Family*:** Fabaceae (also known as Leguminosae)
- ***Chemical constituents***: Glycyrrhizin, glycyrrhetic acid, isoliquiritin, and isoflavones.
- ***Uses***: It has been used for several pharmacological activities like, expectorant, anti-demulcent, anti-ulcer, anti-cancer, anti-inflammatory and anti-diabetic. It is a small perennial herb that has been traditionally used to treat many diseases such as respiratory diseases, hyperdypsia, epilepsy, fever, stomach ulcers, and jaundice.
- ***Plant part used for extraction***: stem or roots were used for extraction.

4. *Cyperus Rotundus*:

- ***Biological source:*** Native to Africa, southern and central Europe, and southern Asia.
- ***Synonyms:*** Nagarmotha, nut grass.

Figure 1.4

- ***Family:*** Sedges (Cyperaceae)
- ***Chemical constituents:*** copaene, cyperene, valerenal, carophyllene oxide, and trans-pinocarveol.
- ***Uses:*** It is a medicinal herb used to treat various diseases such as, diabetes, diarrhea, inflammation, malaria, and stomach, and bowel diseases. In addition, it is cooling in nature, its anti-inflammatory property helps soothe redness, breakouts and inflamed skin. It has proven to treat severe skin conditions. It is rich in fatty acids vitamins, and flavonoids which are extremely beneficial for the skin as well as hair.
- ***Plant part used for extraction:*** rhizomes

Chemical reagents and materials:

- There were four different assays performed for determination of anti-oxidant activity, and the chemical reagents and materials used for the preparation of the solution or in the assays are listed below:

Sr. no	Name of the assay	Reagents and chemicals	Reference
	Hydrogen peroxide scavenging activity	Potassium dihydrogen phosphate Disodium hydrogen phosphate Hydrogen peroxide	**https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8037236/**

	Nitric oxide scavenging activity	Sodium chloride Distilled water Phosphate buffer solution Sodium nitroprusside	**https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8037236/**
	DPPH activity	2,2-dipicrylhydrazyl Ethanol	**https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8037236/**
	ABTS activity	2,2' azino-bis (3-ethylbenzothiazoline) 6-sulfonic acid Ammonium per sulphate Distilled water Ethanol	**https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8037236/**

EQUIPMENT USED:

Sr.no	Equipment
1.	UV spectrophotometer
2.	ELISA

METHODS:

- **Extraction process:**

- All the plants were collected from LVG (Lalubhai Vrijlal Gandhi) store of required quantity (100gm each).
- After that, 50gm of each part plant used for extraction were accurately weighed and 500ml of water was added.
- This was kept for overnight, after 24 hours the solution was heated and then filtered.
- The filtrate was then poured in porcelain dish and heated to get the extract.
- Similarly, four extracts of four different plants were collected in couple of days.
- **Observation table**:

Sr.no	Plant name	Extract	%yield	Consistency
	Brassica Nigra	Aqueous	8.36%	Semi-solid
	Mentha Piperita	Aqueous	2.62%	Semi-solid
	Glycyrrhiza Glabra	Aqueous	12.36%	Solid
	Cyperus Rotundus	Aqueous	6.5%	Semi-solid

ANTI-OXIDANT ACTIVITIES: Two scavenging activities were performed and two chemical assays were performed:

The two chemical assays are as follows:

1. **2, 2-Diphenyl dipicrylhydrazl activity: (DPPH assay)**

- First of all, 0.1mM DPPH solution is prepared by adding 3.94mg in 100ml of ethanol.
- After addition of ethanol, the solution was homogenized for 30 seconds.
- After homogenizing, the solution was incubated in dark for 30 minutes at room temperature.
- The reason for incubating the DPPH solution in dark is because the light would oxidize further the DPPH radical with solution to be determined interfering with your results.
- After that, 96 well micro plates are taken, markings are done according to different concentrations.
- In test, 100µl of each extract of different concentrations were added to their respective well, and 100µl of DPPH solution was added to each well.
- In blank, 100µl of extracts were only added to their respective well plates.
- In control, 100µl of DPPH solution, and 100µl of ethanol was added to their respective well plate.
- Absorbance was taken at 517nm, and results were noted

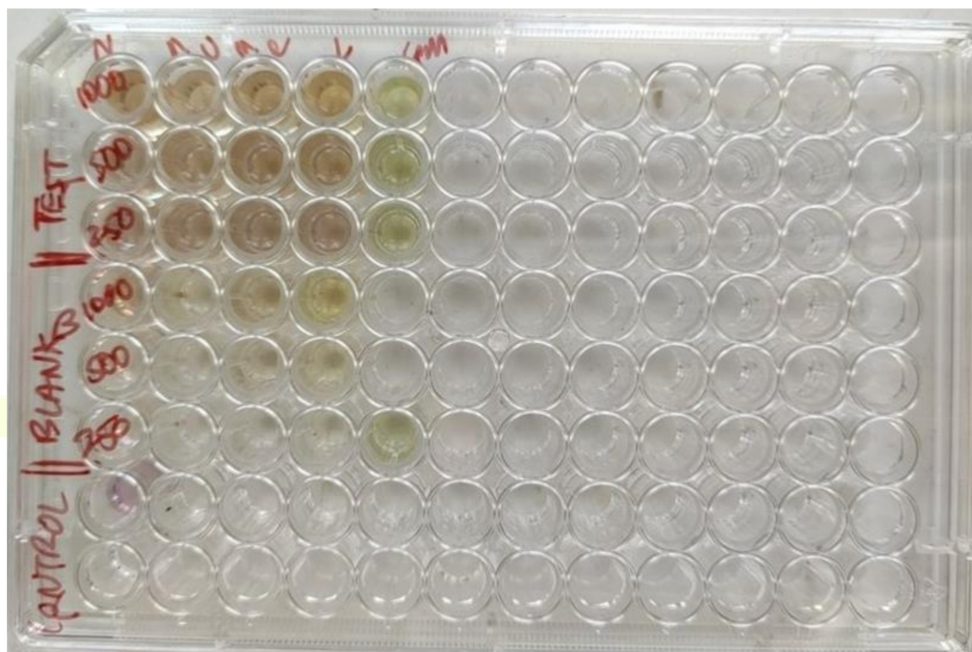


Figure 1.5: DPPH assay

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2. **2, 2'-azino bis 3-ehtylbenzothiazoline-6-sulfonic acid assay: (ABTS assay)**

- First of all, dissolve 0.038gm of ABTS in 10ml distilled water.
- After that 2.45mM ammonium per sulphate solution is prepared by adding 0.559mg in 10ml distilled water.
- After that both the solutions are mixed and allowed to stand in dark at room temperature for 12-16 hours.
- After that 96 well micro plates are taken, markings are done, of different concentrations of each extract.
- In test, 100µl of each extract of different concentrations were added to their respective well plate, and 60µl of ABTS solution was added to each well.
- In blank, 100µl of each extract of different concentrations was added to their respective well.
- In control, 60µl of ABTS solution, and 40µl of ethanol was added.
- Absorbance was taken at 734nm, and results were noted.

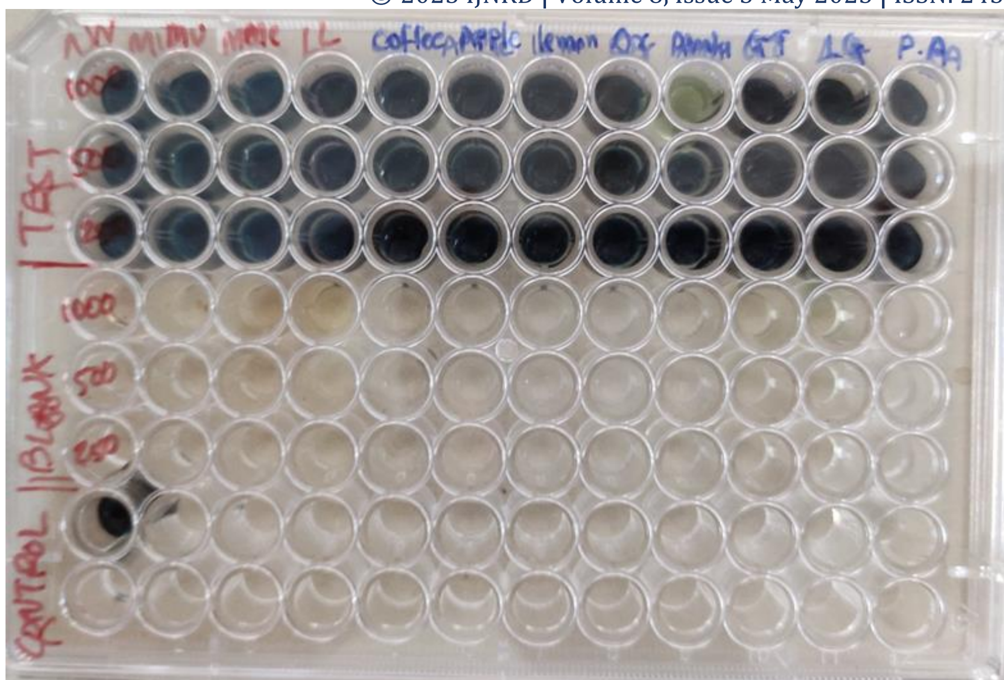

Figure 1.6: ABTS assay

The two scavenging activities are as follows:

3. <u>Hydrogen peroxide scavenging activity</u>:

- Hydrogen peroxide 2mM was prepared by mixing with 50mM phosphate buffer.
- First of all, phosphate buffer was prepared by adding 6.8gm potassium dihydrogen phosphate to 1L distilled water, pH is adjusted with NaOH to make pH 7.4.
- After that, 2mM hydrogen peroxide was prepared by adding 68.02mg of hydrogen peroxide to previously prepared phosphate buffer solution.
- Furthermore, three different concentrations of the extracts were prepared of 1000µg/ml, 500µg/ml, and 250µg/ml.
- For performing the activity, 0.300µl of each extract of each concentration was taken in a test tube, then 1.2µl of buffer was added to each test tube, and 1.8µl of hydrogen peroxide was also added to each test tube.
- The test tubes were allowed to stand for 10 minutes, after that absorbance was taken in UV spectrophotometer, under 230nm wavelength. 1.2µl of buffer and 1.8µl of hydrogen peroxide was taken as blank.
- The results were noted and then % hydrogen peroxide activity was calculated.

4. <u>Nitric oxide scavenging activity</u>:

- First of all, saline solution was prepared by adding 9gm NaCl in 700ml of distilled water and was made up to 1L.
- Then, phosphate buffer was prepared by adding 6.8gm potassium dihydrogen phosphate to 1L above prepared saline solution and pH was adjusted to 7.4 with the help of NaOH.
- Furthermore, 2.98gm of sodium nitroprusside was added to 1L above prepared saline phosphate buffer.
- After that, 96 well microplate was taken, markings were done, as test, blank, and control.
- In test, 30µl of each extract of different concentrations were added to their respective markings, and 30µl of sodium nitroprusside was added to each well.
- In blank, total of 130µl of each extract was added to their respective markings according to their respective concentrations.
- In control, 30µl of each extract to their respective well, and 300µl of Griess reagent was added.

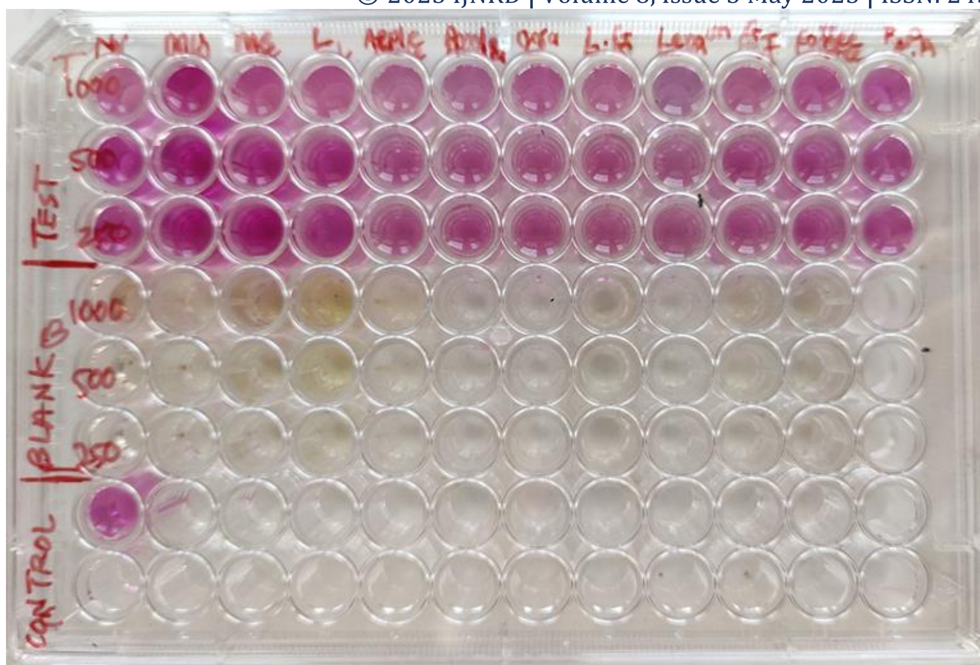

Figure 1.7: NO scavenging activity

RESULT AND DISCUSSION

- **Hydrogen peroxide scavenging activity results:**

Sr.no	plant name	1000µg/ml	500 µg/ml	250 µg/ml
1.	Brassica Nigra	0.006	0.080	0.206
2.	Mentha Piperita	0.175	0.043	0.229
3.	Glycyrrhiza Glabra	0.714	0.241	0.166
4.	Cyperus Rotundus	0.181	0.068	0.234
5.	Control	0.378		

Table 1.6: H2O2 activity result

- **Observation Table:**

	1000µg/ml	500 µg/ml	250 µg/ml
Nagarmotha	53 ORAC	82.1 ORAC	38.1 ORAC
Mentha	53.8 ORAC	89 ORAC	60.5 ORAC
Mustard	98.5 ORAC	21.16 ORAC	45.6 ORAC
Licorice	-	36.24 ORAC	56 ORAC

Table 1.7: H2O2 observation table

- **Calculation:**

$$\% \, ROS \; of \; cyperus \; rotundus \; = 1 - \frac{As}{Ac} * 100$$

$$= 1 - \frac{0.181}{0.378} * 100$$

$$= 1 - 0.47 * 100$$

$$= 0.53 * 100$$

$$= 53 \; ORAC$$

➢ **Discussion:**

- The reported value of ascorbic acid as per table 1.1 is shown to be 50-60 µM, and the calculated value of ***Brassica Nigra*** is **98.5 ORAC which is the highest among all the calculated values**, therefore proving that the plants taken does possess anti-oxidant activity.

➢ **Nitric oxide scavenging activity results:**

	Concentrati-on	Cyperus rotundus	Brassica nigra	Mentha piperita	Glycyrrhiz-a glabra
Test	1000µg/ml	0.674	2.318	0.859	0.729
	500 µg/ml	1.011	1.877	1.082	0.958
	250 µg/ml	0.656	1.779	1.581	0.767
Blank	1000µg/ml	0.143	0.129	0.146	0.115
	500 µg/ml	0.085	0.067	0.100	0.085
	250 µg/ml	0.066	0.069	0.072	0.074
control	0.598				
	0.241				

Table 1.8: NO activity result

➢ **Calculation**:

$$\%ROS\ of\ cyperus\ rotundus = Ac - \frac{As - Ab}{Ac} * 100$$

$$= 0.598 - \frac{0.656 - 0.066}{0.598} * 100$$

$$= 0.598 - \frac{0.590}{0.598} * 100$$

$$= \frac{00.008}{0.598} * 100$$

$$= 1.3\ ORAC$$

➢ **Discussion:**

- The reported value of carotene in table 1.1 is shown to be 0.5-1µM, and the calculated value of ***Cyperus Rotundus*** is **1.3 ORAC**, therefore proving that plant possess anti-oxidant activity.
- Similarly, the calculation of other extracts are performed.

- **DPPH activity results:**

	Concentratio-n	Cyperus rotundus	Brassica nigra	Mentha piperita	Glycyrrhiz-a glabra
Test	1000µg/ml	0.278	0.216	0.274	0.243
	500 µg/ml	0.199	0.179	0.223	0.207
	250 µg/ml	0.180	0.177	0.194	0.202
Blank	1000µg/ml	0.177	0.092	0.136	0.122
	500 µg/ml	0.079	0.081	0.098	0.083
	250 µg/ml	0.072	0.074	0.068	0.067
control	0.171				
	0.049				

Table 1.9: DPPH activity result

- **Observation table:**

	1000 µg/ml	**500 µg/ml**	**250 µg/ml**
Nagarmotha	41.4 ORAC	29.82 ORAC	36.84 ORAC
Mentha	19.3 ORAC	26.9 ORAC	26.31 ORAC
Mustard	27.8 ORAC	42.69 ORAC	39.76 ORAC
Licorice	29.2 ORAC	27.4 ORAC	21 ORAC

Table 2.0: DPPH observation table

- **Calculation:** $\%ROS\ of\ cyperus\ rotundus = Ac - \frac{As-Ab}{Ac} * 100$

$$= 0.171 - \frac{0.278 - 0.177}{0.171} * 100$$

$$= 0.171 - \frac{0.101}{0.171} * 100$$

$$= 41.4\ ORAC$$

- **Discussion:**

- The reported value of ascorbic acid in table 1.1 is shown to be 50-60µM, and the calculated value of ***Brassica Nigra*** in **42.69 which is close about to the reported value and the highest above all the calculated values**, therefore proving the anti-oxidant activity of the plants.

- **ABTS activity result**:

	Concentratio-n	Cyperus rotundus	Brassica nigra	Mentha piperita	Glycyrrhiz-a glabra
Test	1000μg/ml	3.376	3.844	3.290	2.367
	500 μg/ml	3.019	3.450	3.440	2.153
	250 μg/ml	3.432		3.751	2.099
Blank	1000μg/ml	0.072	0.247	0.063	0.061
	500 μg/ml	0.058	0.051	0.050	0.051
	250 μg/ml	0.047	0.045	0.045	0.057
control	3.636				
	0.043				

Table 2.1: ABTS activity result

- **Observation table:**

	1000 μg/ml	**500 μg/ml**	**250 μg/ml**
Nagarmotha	9.13 ORAC	18.56 ORAC	6.9 ORAC
Mentha	11.2 ORAC	6.7 ORAC	2.0 ORAC
Mustard	1.67 ORAC	6.5 ORAC	-
Licorice	36.5 ORAC	42.18 ORAC	43.89

Table 2.2: ABTS observation table

- **Calculation**: $\%ROS\ of\ glycyrrhiza\ glabra = Ac - \frac{As-Ab}{Ac} * 100$

$$= 3.636 - \frac{2.097 - 0.057}{3.636} * 100$$

$$= 3.636 - \frac{2.04}{3.636} * 100$$

$$= 43.8\ ORAC$$

- **Discussion:**

- The reported value of ascorbic acid in table 1.1 is shown to be 50-60μM, and the calculated value of ***Glycyrrhiza Glabra*** is **43.8 which is near about to reported value and the highest among all the calculated values**, therefore proving the anti-oxidant activity of the plant.

- **SUMMARY**

Sr. no	Activity/Assay	Plant- 1st preference	Plant name- 2nd preference
1.	DPPH assay	Brassica Nigra- 42.69 ORAC	Cyperus Rotundus- 41.4 ORAC
2.	ABTS assay	Glycyrrhiza Glabra- 42.18 ORAC	Cyperus Rotundus- 18.56 ORAC
3.	H2O2 activity	Brassica Nigra- 98.5 ORAC	Mentha Piperita- 89 ORAC
4.	NO activity	Cyperus Rotundus- 1.3 ORAC	Cyperus Rotundus- 1.3 ORAC

Table 2.3: Summary

- Thus, from studying above summary it can be clearly seen that ***Brassica Nigra* and *Cyperus Rotundus*** are the two plants which give better anti-oxidant activities in average and compared to other two plants.

HERBAL TABLET FORMULATION:

- First of all, all the above mentioned ingredients as mentioned in table 1.3 were weighed accurately according to required quantity.
- Plant extract was taken 300mg, containing all the four extract in the ratio of **(120mg: 90mg: 60mg: 30mg),** the extracts were triturated until it became free flowing powders.
- After that remaining all ingredients were weighed accordingly and added to mortar and pestle. Now, the whole blend of powder was triturated until it became uniform in nature and free flowing.
- After that, the blend of powder was taken to the rotary machine, where the tablets are being punched.
- Oval dye was taken for punching as the bulk of powder was less, total of 7 tablets were punched each of 500mg.
- After tablets were formulated, three tablet parameters were performed for evaluation such as, hardness of tablet, average weight of tablets, disintegration test.

Sr.no	Ingredients	Quantity taken
1.	Powdered plant extract	300mg
2.	Ethyl cellulose	50mg
3.	Microcrystalline cellulose	50mg
4.	Carbopol	25mg
5.	Methyl paraben	0.2%
6.	Magnesium stearate	15mg
7.	Dibasic calcium phosphate	40mg
8.	PEG 4000	20mg
Total		**500mg/tablet**

Herbal tablet test parameters:

1. **Hardness:** Generally the hardness of the oral tablet is between 5-8kg/cm square. As per I.P there is a criteria decided for the tablets and their standard deviation, which is as below:

Official standards:

As per U.S.P.

Sr.No.	Average weight of tablet	% weight variation acceptable(+ or -)
1.	130 or less mg	(+ or -) 10%
2.	130-324 mg	(+ or -) 7.5%
3.	>324 mg	(+ or -) 5%

As per I.P.

Sr.No.	Average weight of tablet	% weight variation acceptable(+ or -)
1.	84 or less mg	(+ or -) 10%
2.	84-250 mg	(+ or -) 7.5%
3.	>250 mg	(+ or -) 5%

Figure 1.9: IP and USP standards for tablet hardness

- **The hardness of the tablets was found to be about 4kg/cm square, which was determined by Monsanto hardness apparatus**.

2. **Average weight of tablets:** Individual tablets were weighed and the weight was noted down of each tablet, then average weight was calculated as follows:

$$Average\ weight = W1 + W2 + W3 + \frac{W4}{4}$$

$$= 505 + 503 + 501 + \frac{497}{4}$$

$$= \mathbf{501.5}\boldsymbol{mg/tablet}$$

3. **Disintegration time:** In this test, the disintegration time of the tablet is noted. As this is tablet and will be taken by mouth, the tablet will pass through the GIT, thus first 0.1N HCl was prepared, and then it was used for disintegration test. In this test, disintegration apparatus was used to perform the test. The tablet was observed to swell as the time passed by, and started disintegrating, the total disintegration time taken by the tablet to totally disintegrate was around 37 minutes. Thus, this shows that it is sustained release.

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