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Study of In-vitro Antioxidant Activity and Brine Shrimp lethality Bioassay of different Parts of three Cassia Species

Meeta Bhot[1], Meenakshi Barua[2] and Jossy Varghese[3]

[1]Department of Botany, Birla College, Kalyan. Maharashtra
[2]Department of Biotechnology, Birla College, Kalyan. Maharashtra
[3]Department of Botany, Birla College, Kalyan. Maharashtra
E-mail: [1]bhotmeeta@yahoo.co.in, [2]meenakshi.barua@gmail.com, [3]jossy_v@rediffmail.com

Abstract—Free radicals are toxic byproducts of natural cell metabolism and are responsible for causing a wide number of health problems. The use and search of drugs or dietary supplements from plants with antioxidant activity (antioxidants are known as reducing agents) are in great demand. Cassia Linn. (Family-Caesalpiniaceae) are well known in Indian system of medicine for their cathartic, antiparasitic character, anti-helminthic, antifungal, antimicrobial, anti-inflammatory property etc. In the present study, crude aqueous and hydro-alcoholic extracts of different parts (stem, leaves, pods and flower) of Cassia alata, Cassia fistula and Cassia tora were used for In-vitro antioxidant activity and Brine Shrimp lethality assay so as to evaluate their potential medicinal importance. The antioxidant activity of different parts of three species were evaluated by using six methods. The comparative study by using various assays revealed that, both the extracts (aqueous and hydro-alcohol) of Cassia alata were shown satisfactory results than the extracts of remaining selected Cassia plants. Total Polyphenols and flavonoid contents equivalent to cathechol (100µg/ml) and quercetin (100µg/ml) respectively were found higher in hydro-alcohol extracts. The different parts of these plants were further taken for toxicity assessment by the Brine Shrimp lethality assay. The extent of toxicity of the plant extracts were determined based on percentage mortality. The above studies indicated that antioxidant activity and toxicity assay of crude extracts of selected Cassia plants may help in its formulation for new pharmaceutical drug.

Keywords: *Antioxidant Activity, Brine Shrimp Lethality, Cassia alata, Cassia fistula, Cassia tora.*

1. INTRODUCTION

Antioxidants are compounds that play important role in preventing or delaying the onset of major reactive species associated diseases such as aging, cancer, coronary heart disease, Alzheimer's disease, neurodegenerative disorders, atherosclerosis, diabetes, and inflammation (Kaniz Fatima Urmi *et al.,* 2012, HO et al. 2012). Physiologically, these compounds scavenge the free radicals (highly unstable and reactive species) which are capable of damaging molecules such as DNA, proteins and carbohydrates (Kaniz Fatima Urmi et *al.*, 2012). Free radicals are responsible for causing a wide number of health problems which include cancer, aging, heart diseases and gastric problems etc. (Sirappuselvi S., and Chitra, M., 2012). Phenolics are widespread constituents of plant foods (fruits, vegetables, cereals, olive, legumes, chocolate, etc.) and beverages (tea, coffee, beer, wine, etc.), and partially responsible for the overall organoleptic properties of plant foods. These are broadly distributed in the plant kingdom and are the most abundant secondary metabolites of plants. Plant polyphenols have drawn increasing attention due to their potent antioxidant properties and their marked effects in the prevention of various oxidative stress associated diseases (Jin D., and Russell J. M.,2010).

Cassia Linn. (Family–Caesalpiniaceae) is a large tropical genus with about 600 species of herbs, shrubs and trees; some of which widely distributed throughout the world especially in tropical countries and is abundantly available in India. Most of the plants of the genus are well known in Indian system of medicine for their cathartic, purgative antiparasitic, anti-helminthic, antifungal, antimicrobial, anti-inflammatory property etc. Some of plants of genus are widely used as traditional medicine in Africa and India for the treatment of ulcers. Most of the plants are also used traditionally in the treatment of periodic fever and malaria in subtropical and tropical regions. Several of them yield timber, dyes, fodder, vegetables, edible fruits, leaves and seeds where in some places seeds are used as substitute for coffee (Dave, H. and Ledwani L., 2012). Plants of *Cassia* genus are rich source of polyphenols, flavonoids, tannins, mucilage, polysaccharide, steroids, anthraquinone glycosides and anthracene derivatives (Sanghi *et al.,* 2006). The anti-inflammatory activity of *Cassia* may be attributed to the flavonoid molecules present in them (Ganapaty *et al.,* 2002).

The aim of this comparative study of different parts (stem, leaves, pods and flower) of *Cassia alata*, *Cassia fistula* and *Cassia tora* is to determine the *In-vitro* antioxidant activity of

crude aqueous and hydro-alcoholic extracts *via* using various reducing assays *viz.* DPPH, ABTS/TEAC (Trolox equivalent antioxidant capacity), Phosphomolybdate and Nitric Oxide scavenging activity to emphasis on a potential source for bioactive molecules. Total Phenolic and flavonoid content of fractions were also determined in order to evaluate a relationship between the antioxidant activity and phytochemical constituents. Furthermore toxicity of different extracts of various parts were investigated by Brine Shrimp lethality bioassay to evaluate their potential medicinal importance.

2. MATERIALS AND METHODS.

2.1 Materials

Three plants namely *Cassia alata, Cassia fistula* and *Cassia tora* were collected from Suburban and urban region of Maharashtra, India. They were identified and authenticated from the Blatter Herbarium, St. Xavier's College, Mumbai.

All chemicals used in assays were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Merck Co. (Santa Ana, CA, USA).

Brine Shrimp Eggs (San Francisco Origin, Artemia Cysts) were purchased from Artemia International LLC, USA.

Methods

Preparation of the extracts and fractions.

The air-dried powdered stem, leaves, pods and flower of *C. alata, C. fistula and C. alata* (100 g) was extracted with water and hydroalcohol (ethanol-water (1:1)) mixture using a mechanical shaker for 12-18h. The resultant extract was concentrated under reduced pressure to yield a residue. Then the crude aqueous and hydro-alcholic extract were filtered and fractions were used for further analysis.

In-vitro antioxidant activity of fractions were determined by performing six different methods and toxicity assay was evaluated via Brine Shrimp bioassay.

2.2 Antioxidant assays were performed via following different methods

2.2 a) DPPH free radical Assay.

The free radical scavenging activity of the fractions was measured *in vitro* by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (Mensor *et al.,* 2001). 0.2 mM solution of DPPH in ethanol was added to the fraction of aliquot at concentrations (100 µg/ml). The mixture was allowed to stand for 30 min and the absorbance was measured at 517 nm using a UV-Visible spectrophotometer. The % scavenging activity was determined and the IC_{50} value of the fractions was compared with ascorbic acid and trolox (10 - 100µg/ml), which were used as the standard.

2.2. b) ABTS/TEAC free radical Assay

A Trolox equivalent antioxidant capacity (TEAC)/ABTS assay was conducted based on the method of Ramos *et al.* (1999). The ABTS solution (7mM) was oxidized with potassium peroxodisulfate (2.45 mM) for 16–18hours at room temperature. The ABTS solution was diluted with solvents. An aliquot (100 µg/ml) was mixed with diluted ABTS solution and the absorbance was read at 734 nm. Trolox and ascorbic acid were used as reference standards. TEAC value was expressed as millimolar concentration of trolox and ascorbic acid solutions, with the antioxidant equivalent to a 1000 ppm solution of the sample.

2.2. c) Nitric Oxide scavenging Assay

This assay was performed according to the method described by Sreejayan *et al.,* (1997). Nitric oxide generated from sodium nitroprusside at physiological pH interacts with oxygen to produce nitrite ions, which was measured by Griess reagent. The 10 mM sodium nitroprusside solution and the fractions or the reference compound at different concentrations were incubated for 150 min. Then 0.5 ml aliquot of the incubated sample and 0.5 ml Griess reagent was added. The absorbance was measured at 546 nm. Inhibition of the nitric oxide generated was measured by comparing the absorbance values of control, fractions and ascorbic acid as a reference compound.

2.2. d) Phosphomolybdate Assay

The total antioxidant capacity of the fractions was determined by Phosphomolybdate method using ascorbic acid as the standard (Jayaprakasha *et al.,* 2002). An aliquot of 0.1ml of the fractions (100µg) solution was combined with 1ml of reagent (0.6M sulphuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate). The tubes were incubated at 95ºC for 90 min. The absorbance was measured at 695 nm using an UV-Vis spectrophotometer. The total antioxidant capacity was expressed as µg equivalents of ascorbic acid by using the standard graph.

2.2. e) Folin-Ciocalteu Method

Total soluble phenolics of the fraction were determined with Folin-Ciocalteu reagent using catechol as the standard (Gulcin *et al.,* 2004). An aliquot of 0.1 ml of sample was used for analysis with Folin-Ciocalteu reagent, followed by 2% sodium carbonate was added. Subsequently, the mixture absorbance was measured at 760nm. The concentration of total phenolic compounds in the fractions was determined by using the standard catechol graph.

2.2. f) Total Flavonoid Content

Total soluble flavonoid content was determined by using modified Aluminium Chloride ($AlCl_3$) Colorimetric method using quercetin as a standard (Hsu, 2006). Each plant extract was mixed with 0.1ml of $AlCl_3$, 0.1ml of potassium acetate and distilled water. Then incubated at room temperature for 30

minute. The absorbance of reaction mixture was measured at 415nm. The calibration curve was plotted by preparing the quercetin solution at concentration from 10 to 100µg/ml.

2.3 Brine Shrimp Bioassay.

Artemia Cyst Processing, Hatching and Incubation Procedure.

Artemia cysts were processed via decapsulation of outer membrane called the chorion by dissolving them in sodium hypochlorite. Then hatched them in sterilized container with seawater at standard condition (30 ± 1ºC. 1,500 lux light intensity). Aeration was maintained by a small pipe extending to the bottom of the hatching device via an aquarium air pump. After 24-38hrs set them for incubation in seawater with sample (1000ppm) under the same standard condition. Morality rate was determined after 24hrs of incubation with sample.

Percentage mortality was calculated by following the Formulae:

$$\% \text{ Mortality} = \frac{\text{Number of dead } \textit{Artemia nauplii}}{\text{Initial number of live } \textit{Artemia nauplii}} \times 100$$

3. RESULTS AND DISCUSSIONS

3.1 a) DPPH free radical Assay.

The DPPH antioxidant assay is based on the ability of DPPH to decolorize in the presence of antioxidants. In case of antioxidant screening (Fig. 1.a,b) the hydro-alcoholic extract of parts showed the highest antioxidant activity. At the same time, the hydro-alcoholic extract of *Cassia alata* also exhibited signifiant antioxidant activity (IC50=(L-28.16;S-9.31;P-2.24,F–25.17µg/ml), where standard Ascorbic acid and Trolox showed free radical scavenging with the IC50 value 22.78 µg/ml. All the extracts showed a dose dependent scavenging activity of DPPH comparable to standard antioxidants. These results denote the presence of antioxidant principles in the extractives.

3.1 b) ABTS/TEAC free radical Assay.

A comparison of the TEAC values of antioxidants obtained by the ABTS persulfate decolorization assay is shown in Figure 2.2. a & 2.2 b. The extract of *Cassia alata* was showed highest ABTS scavenging activity in both the extracts (aqueous and hydro-alcohol). Free radical scavenging activity of aqueous extracts was found higher than hydro-alcohol (Fig. 2.2. **b)**. Comparatively leaves of the three species in both the extracts found moderately high. All the extracts showed a dose dependent scavenging activity of ABTS comparable to standard antioxidants. Fig. 2.2. **a** shows ABTS radical scavenging activity of standard ascorbic acid and trolox.

3.1 c) Nitric Oxide scavenging Assay.

Nitric oxide is a very unstable species under the aerobic condition. It reacts with Oxygen to produce the stable product nitrates and nitrite through intermediates through NO_2, N_2O_4 and N_3O_4. It is estimated by using the Griess reagent. In the presence of test compound, which is a scavenger, the amount of nitrous acid will decrease. The extent of decrease will reflect the extent of scavenging; the percentage inhibition of hydro-alcholic extract showed that IC50 values *Cassia alata* L-50.34, S-35.78, P-82.32, F-98.16 µg/ml. The standard of Ascorbic acid showed highest inhibition % in hydro-alcoholic extract (Aq-56.66%, H.A-61.31%) which is shown in Figure 3.a) and Fig. 3.b).

3.1 d) Phosphomolybdate Assay.

The phosphomolybdate method is quantitative, since the total antioxidant capacity is expressed as ascorbic acid equivalents. Among the fractions tested, the hydroalcoholic fraction of *Cassia tora parts* contains moderately higher concentration (µg) of ascorbic acid equivalent/100 µg than other parts of *Cassia* plant. The antioxidant activity of Aqueous extracts showed moderately same to hydroalcholic extracts of different parts of *Cassia* Species which is shown in Fig. 4.a & Fig. 4.b.

3.1 e) Total Phenolic Content (Folin-Ciocalteu Method) and Total Flavonoid Content.

Total phenolic content was estimated by using Folin-Ciocalteu reagent. Total phenolic content of the different fractions of *C. alata, C.fistula and C. tora* were solvent dependent and expressed as µg Catechol equivalent. The total phenolics content in the hydroalcoholic fractions showed higher concentration than aqueous. Maximum flavonoid content found in hydroalcoholic extracts of leaves and pods of *Cassia alata* (Fig. 5.a & Fig. 5.b). The total flavonoid content in the fractions was expressed *C. alata, C.fistula and C. tora* as µg quercetin equivalent. The hydro-alcoholic fractions of different parts showed highest amount of flavonoids than the fractions of aqueous. The content of total flavonoids in the fractions decreased in the order of flowers > leaves> pods > stem (Fig. 6.a & Fig. 6.b).

3.2 Brine Shrimp Assay.

This study revealed that the hydro-alcohol extracts of different parts of *Cassia* species have less number of mortality than aqueous extracts Fig.7. Less number of alive *Artemia nauplii* was observed in aqueous extracts of *Cassia tora.* Satisfactory number of alive *Artemia nauplii (s)* were observed in positive control whereas 100% mortality was found in negative control. Less mortality % was found in extracts of *Cassia alata* except the aqueous extracts of leaves of *Cassia alata.* 100% mortality was observed in leaves of *Cassia fistula* and flower of *Cassia tora.* This assay helped in evaluation of best extracts of different *Cassia* species.

(** Note- Aq- Aqueous ; H.A–Hydro-alcohol; L- Leaves, S-Stem; P-Pods, F- Flower; 1- Cassia tora, 2- Cassia fistula, 3- Cassia alata)

3.3 Figures and Tables.

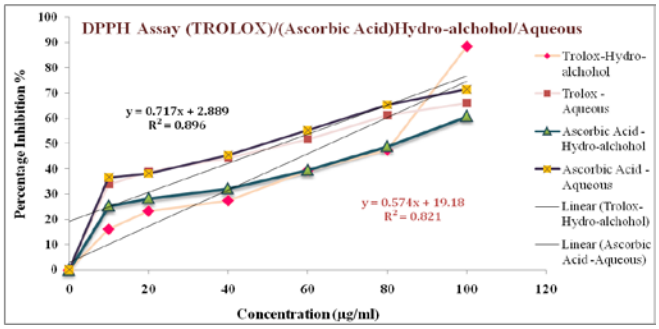

Fig. 1 a) DPPH Assay - Standard

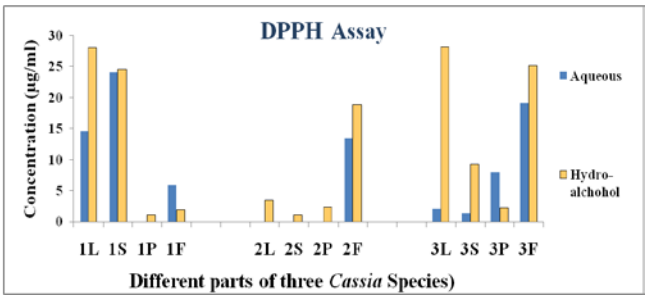

Fig. 1 b) DPPH Assay

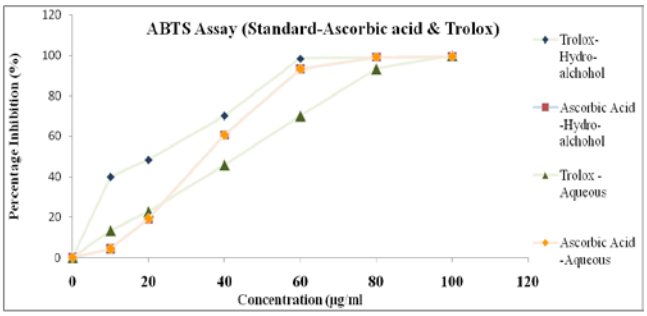

Fig. 2 a) ABTS Assay–Standard.

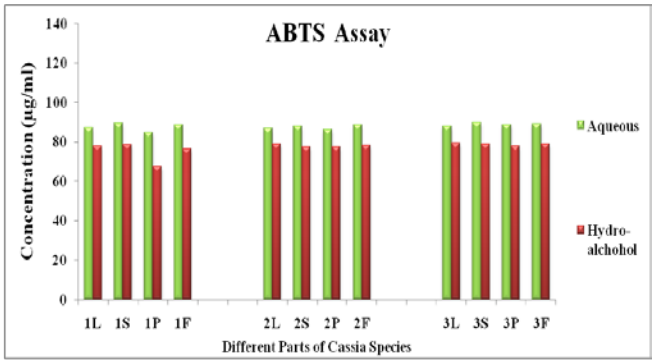

Fig. 2 b) ABTS Assay.

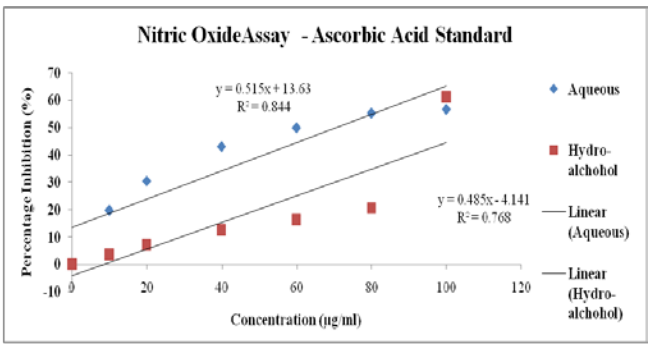

Fig. 3 a) Nitric Oxide Assay–Standard.

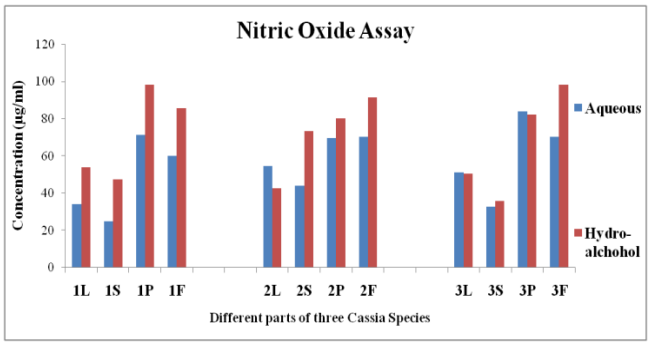

Fig. 3 b) Nitric Oxide Assay.

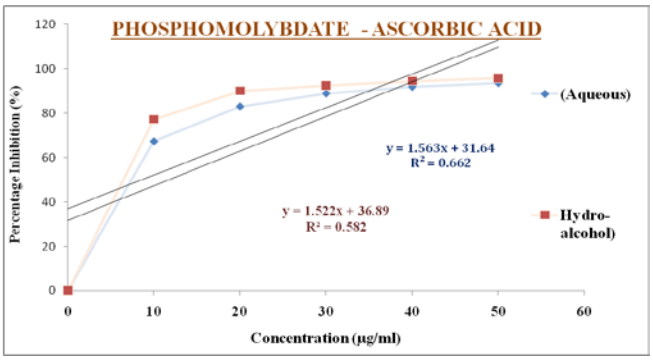

Fig. 4 a) Phosphomolybdate Assay–Standard.

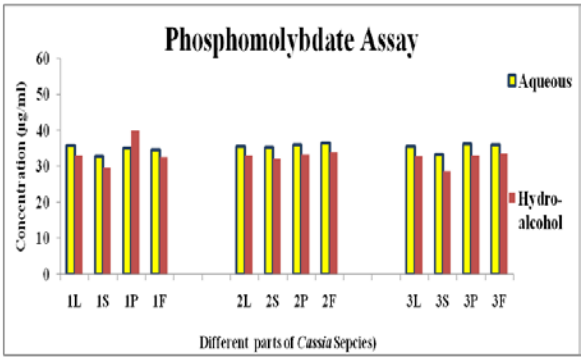

Fig. 4 b) Phosphomolybdate Assay.

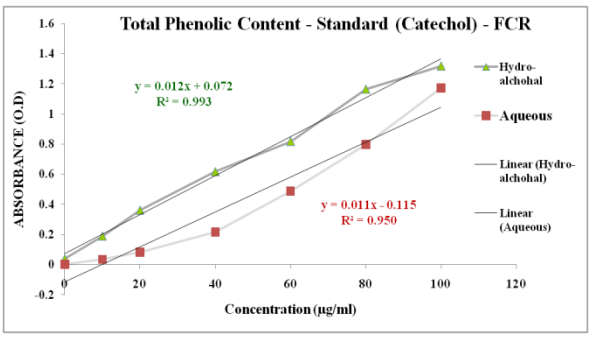

Fig. 5 a)Total Phenol Content (FCR)– Standard.

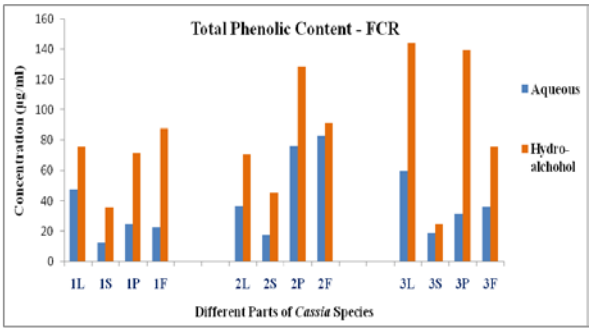

Fig. 5 b)Total Phenol Content (FCR).

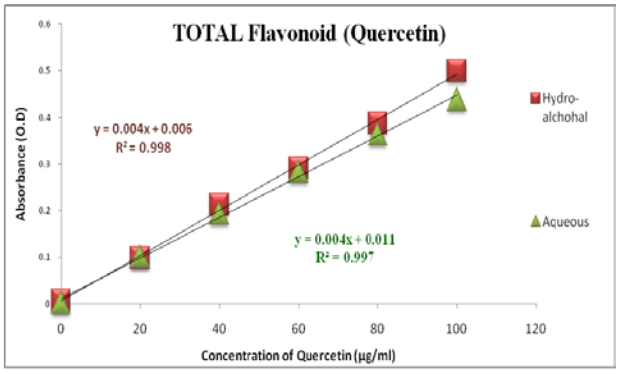

Fig. 6 a)Total Flavonoid Content ($AlCl_3$)– Standard.

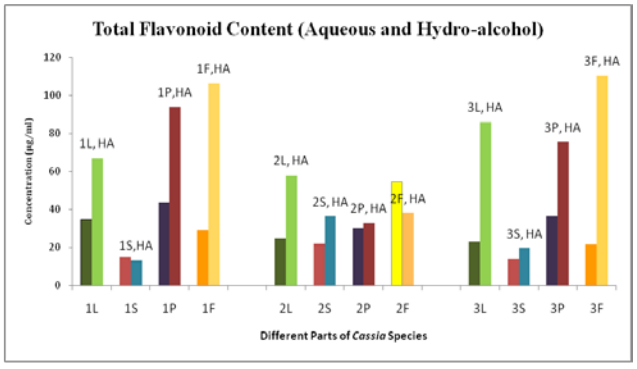

Fig. 6 b) Total Flavonoid Content ($AlCl_3$).

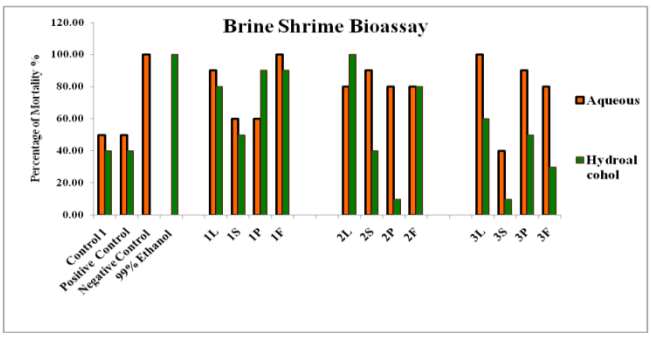

Fig. 7: Brine Shrimp Bioassay

4. CONCLUSIONS

In conclusion, the results of the present study suggest that *Cassia alata*, *Cassia fistula* and *Cassia tora* plant materials have potent antioxidant activity. The providing data reveled about the significant relation between the amount of phenolics and flavonoid contents and free radical scavenging activity. The given data can enrich the existing comprehensive data of free radical scavenging activity of plant materials of selected *Cassia* species. It is very much helpful for investigation of new drugs for various free radical generation diseases by identifying the bioactive compounds in different extracts.

5. ACKNOWLEDGEMENTS

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