Isolation and anti-oxidant activity of natural pigments from *Hibiscus rosa sinensis (Malvaceae)* and *Tagetes erecta (Asteraceae)*

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***Abstract*—Natural dyes and pigments are produced by living organisms such as plants, animals, fungi, and microorganisms. Dyes and Pigments are chemical compounds that absorb light in the wavelength range of the visible region. The need for dyes and pigments has increased according to the human desire for colors in the required amounts used in food, medicine, textiles, etc. The main objective of this study was to isolate natural pigments from *Hibiscus rosa sinensis* (*Malvaceae*) and *Tagetes erecta* (*Asteraceae*) and predict of antioxidant compounds from plants. The ethanolic extracts were used for the isolation and separation of antioxidant compounds using different chromatographic techniques, such as column chromatography, thin layer chromatography, and spectroscopic analytical methods. The antioxidant activity of the isolated pure compounds was determined using the modified 1,1-diphenyl-2-picrylhydrazyl (DPPH) and reducing power methods. The main mechanism of action of antioxidants is to improve the immune system in the human body by preventing the formation of free radicals. The isolated pure pigment fractions obtained from the ethanolic extracts of the selected plant species could be a good source of natural antioxidants.**

***Keywords*—Antioxidant, Chromatography, *Hibiscus,* Pigments. *Tagetes erecta.*. etc.**

I. INTRODUCTION

Plants are considered an essential source of medicines for humans. Plant parts like leaves, flowers, roots, stems, seeds, and fruit are used as food resources for human as well as safe medicine for the treatment of different diseases.[1] Plant-derived herbal medicine is used as main sources for the treatment of diseases since the ancient time.[2]Colour of a food substance is important to indicate its freshness and safety that are also indices of good aesthetic and sensorial values.[3] In the recent years, colouring of food with pigments produced from natural sources is of worldwide interest and is gaining importance.[4] These pigments are looked upon for their safe use as a natural food dye in replacement of synthetic ones because of undesirable market.[5]

Well-textured food rich in nutrients and flavor cannot be eaten unless it has the right color. Color additives have long been used as a means of enhancing the aesthetic value of foods, beverages, and cosmetics, and for identifying drugs and other products.[6] The demand for natural sources of such compounds is increasing day by day because of the awareness of positive health benefits from natural compounds.[7]Humans without color blindness can detect wavelengths between approximately 380 and 730 nm, representing the visible spectrum of red, orange, yellow, green, blue, indigo, and violet. Therefore, chlorophyll with maximum absorbance at 430 and 680 nm leaves wavelengths that form a green color. Often the colours are the result of a mix of residual wavelengths; for example, anthocyaninsm absorbing yellow-green light wavelengths of 520–530 nm will generate mauve colours formed by the reflection of a mix of orange, red and blue wavelengths.[8] Thus the pigments can be described in two ways: the wavelength of maximum absorbance (lmax) and the colour perceived by humans.[9]

Pigments produce colors that we observe at each step of our lives because pigments are present in each of the organisms in the world, and plants are the principal producers. They are present in leaves, fruits, vegetables, and flowers. They are also present

in the skin, eyes, and other animal structures, as well as in bacteria and fungi. Natural and synthetic pigments are used in medicines, foods, clothes, furniture, cosmetics, and in other products.[10] However, natural pigments have important functions other than the imparted beauty, such as the following: we could not have photosynthesis or probably life all over the world without chlorophylls and carotenoids.[11] Antioxidants are the most important chemical components that may prevent or delay different types of cell damage. Foods, fruits, and vegetables are the primary sources of natural antioxidants. The prevention or recovery of cell damage in the human body can be achieved by using natural antioxidants in foods, fruits, and vegetables. The main mechanism of action of antioxidants is to improve the immune system in the human body by preventing the formation of free radicals.[12] Some natural antioxidants are also available as dietary supplements. Recently, vegetables and fruits have been considered rich sources of natural antioxidants. Several recent studies have shown that eating many vegetables and fruits is a very good source for human health and to prevent cell damage. Therefore, the risks of certain chronic or incurable diseases are lowered due to natural antioxidants.[13]

Hibiscus rosasinensis is known in China and belongs to the *Malvaceae* family. This plant has various important medicinal uses for treating wounds, inflammation, fever and coughs, diabetes, infections caused by bacteria and fungi, hair loss, and gastric ulcers in several tropical countries.[14] Phytochemical analysis documented that the main bioactive compounds responsible for its medicinal effects are namely flavonoids, tannins, terpenoids, saponins, and alkaloids. Experiment from recent studies showed that various types of extracts from all *H. rosasinensis* parts exhibited a wide range of beneficial effects such as hypotensive, anti-pyritic, anti-inflammatory, anti-cancer, antioxidant, anti-bacterial, anti-diabetic, wound healing, and abortifacient activities.[15]

Tagetes erecta Linn. is a marigold belonging to the *Asteraceae* family. The flowers of *Tagetes erecta Linn*, traditionally used in ancient times, are used in folk medicine to treat various diseases. Leaves are used as antiseptic agents and are also used to treat kidney troubles, muscular pain, piles, and boils. Preliminary evaluation revealed that *Tagetes erecta Linn* flowers contain phytoconstituents such as tannins, phenolic compounds, Flavonoids, sterols, triterpinoids, saponins and alkaloids.[16] The flowers are used to cure fever, epileptic fits according to Ayurveda, astringent, carminative and stomachic, scabies and liver complaints and also employed in diseases of the eyes. They are said to purify blood and flower juice is given as a remedy for bleeding piles and is also used in colds, rheumatism and bronchitis.[17]

II. MATERIALS AND METHODS

2.1. Chemicals and reagents

Solvents such as hexane, ethyl acetate, butanol, chloroform, acetone methanol, silica gel, preparative TLC, Gallic acid, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were used in this experiment obtained from Molychem (Mumbai, India). All other chemicals used were of analytical grade. All glassware used in this study was obtained from Borosil, India.

2.2. Instruments

The absorbance of extracts with various polarities and isolated pure compounds at different concentrations was measured by UV–visible spectroscopy (Shimadzu, Model 1800, Japan) for the determination of antioxidant activity.

2.3. Sample collections:

Hibiscus rosa sinensis (*Malvaceae*) (Fig.1) and *Tagetes erecta* (*Asteraceae*) (Fig.2) flowers were collected from Annamacharya medicinal garden, Annamaiah dist, and Andhra Pradesh. The plants were authenticated by Dr. J. Kamakshamma, Professor and Head, Department of Botany, S.V. University, Tirupathi. Voucher specimens (2023/632) and (2023/740) of the plants were deposited in a college. Fresh flowers were collected and shade-dried for seven days.

Fig 1: *Hibiscus Rosa Sinensis.*

Fig 2:*Tagetes erecta*

2.4. Extraction

2.4.1. Sample Collection and Preparation

Fresh flowers of *Hibiscus rosa sinensis* and *Tagetes erecta* were collected and cleaned with distilled water to remove any dirt or impurities. The flowers were dried by shade drying at a controlled temperature (32°C) to prevent pigment degradation. After drying, the samples were ground into a fine powder.

2.4.2. Solvent Extraction:

The powder samples of *Hibiscus rosa sinensis* (200 g) were extracted with ethanol (300 ml) using a continuous hot percolation method for 6 h. Then.[18] The solvent was evaporated from the extract using a rotary evaporator under reduced pressure at 35 °C. The solvent-free extract (10 g) was acidified with 1% HCl, dissolved in water (200 ml), filtered, and evaporated under reduced pressure using a rotary evaporator, leaving behind the pigment extract (1.64 g). The crude pigment extract was separated and identified using column and thin-layer chromatography.[19]

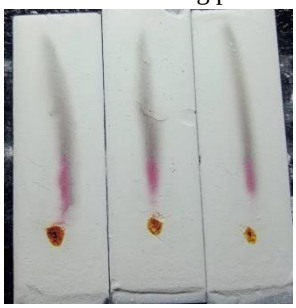
A powder sample of *Tagetes erecta* (200gm) was extracted with ethyl acetate (300ml) using a continuous hot percolation method for 4 h at 40 °C. The solvent was evaporated from the extract by rotary evaporation under reduced pressure at 40 °C. The crude extract yield of 2.35 g was used for further purification. The crude pigment extract can be separated and identified the fractions using column chromatography and Thin Layer Chromatography.

The crude extracts of *Hibiscus rosa sinensis* (1.64 g) and *Tagetes erecta* (2.35 g) were separated using column chromatography with hexane-ethyl acetate (1:1) as the mobile phase. The isolated fractions from the column chromatography were combined together and evaporated the mother solvent at ambient temperature.[20] Based on the TLC, these similar TLC pattern eluents were combined together to give Fraction 1, Fraction 2. According to TLC, two fractions (fraction 1 and fraction 2) were obtained from the crude extracts of *Hibiscus rosa sinensis*(1.64 g) and *Tagetes erecta* (2.35 g). All the fractions were evaporated at ambient temperature inside a fume hood.

The isolated fraction was purified by preparative thin-layer chromatography (PTLC) to obtain pure compounds. Fractions 1 and 2 were further purified by PTLC over silica gel 60 G using hexane-ethyl acetate (1:1) as the developing solvent. Fraction 1 yielded one major compound with several minor compounds.[21] The separated major compound was crystallized from ethyl acetate-chloroform to give a red powder (5.4 mg), Rf 0.44 (hexane–ethyl acetate; 1:1). Fraction 2 produced one major compound and several minor compounds. The separated major compound was crystallized from ethyl acetate-chloroform to give a yellowish powder (3.8 mg), Rf 0.56 (hexane–ethyl acetate; 1:1). Column chromatography leads to a higher resolution than other purification methods and is thus more efficient in terms of the purification of phytoconstituents.[22]

2.5. Thin layer chromatography

Modern TLC mainly exists as a complementary technique to other column-based liquid chromatographic methods to provide additional separation information (multimodal separation techniques). TLC plays a crucial role in the early stage of drug development when information about the impurities and degradation products in drug substance and drug product is inadequate.[20]



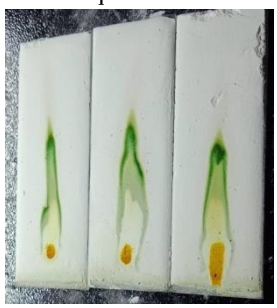


Fig.3: Isolated fractions of *Hibiscus*
Fig.4: Isolated fractions of *Tagetes*

2.5.1. Antioxidant activity by DPPH method

The antioxidant activity of different crude extracts and isolated pure compounds from the selected plants was estimated by 1 the 1-diphenyl-2-picryl-hydrazyl (DPPH) method[23] with modifications. Various concentrations (20, 40, 60, 80, and 100 mg/ml) of each crude extract and pure compound were used to determine antioxidant activity. Each concentration of crude extract (4 ml) was placed in a clean test tube, and DPPH solution (1 ml) was added to the same test tube. The mixture was shaken vigorously by hand and maintained in the dark at

ambient temperature for 45 min. DPPH and methanol was used as the blank samples. The absorbance of all working samples was measured at a fixed wavelength of 517 nm using a UV–visible spectrophotometer,[23] and the percentage of inhibition of each concentration crude plant extract was calculated using the following formula:

2.5.2. Reducing Power Method

The reducing power assay method was used to determine antioxidant activity. In this 1 ml of plant extract of Ethanolic extracts of *Hibiscus rosa sinensis* (EEHR) And *Tageste erecta* (EETE) mixed with 2.5 ml phosphate buffer (0.2M, pH 6.6) and 2.5 ml Potassium Ferricyanide [K_3Fe (CN6)] (10g/l), the mixture was incubated at 50°C for 20 minutes. 2.5 ml of tri chloroacetic acid (100g/l) was added to the mixture. The mixture was centrifuged at 3000 rpm for 10 min. Finally, the supernatant (2.5 mL) was mixed with distilled water and 0.5 ml $FeCl_3$ (lg/L), and the absorbance was measured at 700 nm using a UV-visible spectrophotometer (SHIMADZU UV-1700, UV-visible spectrophotometer). Ascorbic acid was used as the standard and phosphate buffer was used as a blank.

III. RESULTS

The percentage yields of various extracts of *Hibiscus rosasinensis* (*Malvaceae*) and *Tageteserecta* (*Asteraceae*) are presented in Table 1. The ethanol extract gives the high percentage yield and it was found to be 24.60% w/w and 27.30% respectively.

Preliminary phytochemical screening of *Hibiscus rosasinensis* (*Malvaceae*) was performed using different extracts. The results of the phytochemical screening of various extracts are presented in Table 3. The ethanol extracts of *Hibiscus rosa sinensis* (*Malvaceae*) and *Tagetes erecta* (*Asteraceae*) contain constituents such as carbohydrates, anthraquinone glycosides, saponins, tannins, gums, mucilage, and flavonoids. The ethanol extract yielded a high percentage of the yield. Hence, we selected ethanol as the solvent for the extraction and other evaluation steps.

Table 1:Percentage yield of *Hibiscus rosasinensis* and *Tageteserecta*

Plant name	Part used	% yield of extractive (%w/w)		
		Ethanol	Pet. ether	Water
Hibiscus rosasinensis	Flowers	24.60	12.50	10.4
Tageteserecta	Flowers	27.30	9.70	12.0

Table 3: Phytochemical screening of the extracts of *Hibiscus rosa sinensis* and *Tagetes erecta*

Tests	*Hibiscus rosa sinensis*		*Tagetes erecta*	
	Ethanol	Water	Ethanol	Water
Alkaloids	–	–	–	–
Carbohydrates	+	+	+	+
Glycosides	–	+	+	+
Phytosterols	–	–	+	+
Fixed Oils & fats	+	+	+	+
Saponins	+	+	+	+
Phenolics &Tannins	+	+	+	+
Proteins	–	–	–	–
Gums & mucilage	+	+	+	+
Flavonoids	+	–	+	+

Table 4: Antioxidant activity by DPPH method

S. No.	Conc. (µg/ml)	Absorbance of ascorbic acid	Absorbance of EEHR	Absorbance of EETE	%scavenging DPPH of Ascorbicacid	%scavenging DPPH of	%scavenging DPPH of
1	20µg/ml	0.142	0.128	0.121	35.15	41.55	38.55
2	40µg/ml	0.102	0.089	0.098	53.42	59.36	57.34

3	60µg/ml	0.086	0.067	0.079	60.73	69.40	67.88
4	80µg/ml	0.058	0.039	0.042	73.51	82.19	79.32
5	100µg/ml	0.032	0.011	0.025	85.38	94.97	96.35

Table 5: Antioxidant activity by reducing power method

S. No.	Conc. (mg/ml)	Absorbance of Ascorbic acid	Absorbance of EEHR	Absorbance of EETE
	0.1	0.16	0.11	0.14
2	0.2	0.22	0.20	0.21
3	0.3	0.31	0.28	0.27
4	0.4	0.39	0.35	0.36
5	0.5	0.48	0.41	0.42

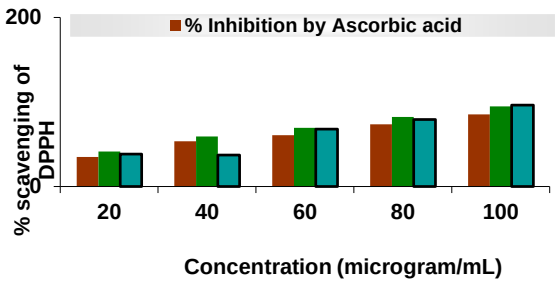


Fig. 5: Antioxidant activity by DPPH method

Fig. 1: DPPH radical scavenging activity of ethanolic extracts of *Hibiscus rosasinensis* (EEHR) And *Tagetes erecta* (EETE) added to an alcohol solution of DPPH, and radical scavenging activity was measured at 517 nm as compared to standard ascorbic acid. Values represent the average of triplicate experiments.

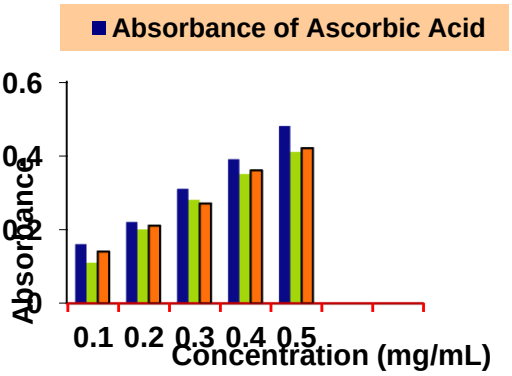


Fig. 6: Antioxidant activity by reducing power method

Fig. 2: Reducing power of ethanolic extracts of *Hibiscus rosa sinensis* (EEHR) And *Tagetes erecta* (EETE) compared with ascorbic acid. Values are the average of triplicate experiments.

IV. DISCUSSION AND CONCLUSION

Many medicinal plants have been investigated worldwide for their biological activity. Most researchers are interested in identifying new naturally occurring drugs with significant medicinal value, without side effects, for the treatment of numerous diseases. Crude plant extracts contain several chemical compounds that are responsible for their biological activities. The bioactivity of crude extracts obtained from plants depends completely on their bioactive constituents. The selected medicinal plant species showed several biological activities, such as antioxidant,[18] antimicrobial, anticancer, antidiabetic, antifungal, and antidiarrheal activities, and are also traditionally used for the treatment of different diseases.

The plants were extracted using continuous hot percolation in a Soxhlet apparatus. The percentage yields of methanol, ethanol, petroleum ether, and aqueous extracts of *Hibiscus rosa sinensis* (*Malvaceae*) and *Tagetes erecta* (*Asteraceae*) were evaluated. Preliminary phytochemical screening of the plant showed positive results for glycosides, saponins, tannins, gums, mucilage, and flavonoids.

In this study, ethanolic extracts of *Hibiscus rosasinensis* (EEHR) And *Tageste erecta*(EETE) were investigated using DPPH scavenging and reducing power assays. The Ethanolic extracts of *Hibiscus rosa sinensis* (EEHR) And *Tagetes erecta* (EETE) showed by their two methods effectively when compared with reference standard ascorbic acid. In Fig.7 the DPPH scavenging method is based on the ability of DPPH radicals to decolorize in the presence of antioxidants. DPPH radical is considered a model of a stable lipophilic radical chain reaction. In lipophilic radicals initiated by lipid autooxidation, antioxidants react with DPPH, reducing the number of DPPH molecules to be equal to the number of hydroxyl groups. Therefore, the absorption at 320 nm was proportional to the amount of residual DPPH.[20] The EEHR and EETE exhibited a significant dose dependent inhibition of DPPH activity, the IC50 value of the EEHR and EETE and reference standard ascorbic acid were found to be 32 mg/mL and 38 mg/mL, 37 mg/mL respectively.

The reducing power method is based on the capability of reducing the compound owing to the presence of reductants that break the free radical chain by donating hydrogen atoms. The plant extracts of EEHR and EETE exhibited antioxidant activity owing to the presence of reductants (i.e., antioxidants). The reduction of Fe^{3+}/Ferricyanide complex to the ferrous form, in this main principle, increases the absorbance of the reaction mixture, which indicates the antioxidant activity that leads to the reducing power of the samples. EEHR and EETE were very potent, and the power of the extract increased with increasing quantity of the sample. By comparison with the reference standard, ascorbic acid.

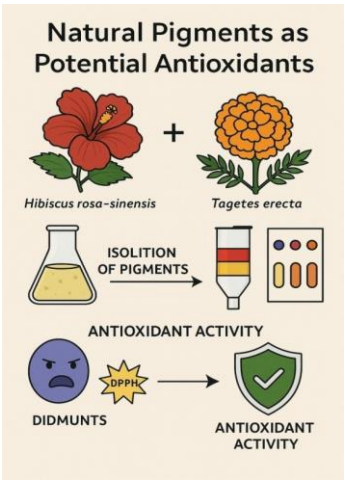


Fig.7: Natural pigments as potential antioxidants

V. CONCLUSION

It is concluded from the data, The pigments extracted from *Hibiscus rosa-sinensis* and *Tagetes erecta* are potent sources of natural antioxidants, contributing to their potential health benefits. These isolated pigments can be further explored for their applications in food, cosmetics, and pharmaceuticals as natural colorants and antioxidants. Ethanolic extracts of *Hibiscus rosa sinensis* (EEHR) And *Tagetes eerecta* (EETE) possess significant antioxidant activity and may prove to be effective for the treatment of various diseases caused by free radicals. The antioxidant activity of this plant may be rich in flavones, anthocyanins, and tannins. Further analytical studies are required for the isolated fractions 1 and 2 ther to structural characterization of active compounds, and an in vivo study is required to confirm the mode of biological activities as well as the structure-activity relationship.

Disclosure statement

The authors declare no potential conflicts of interest.

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