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<i>e-ISSN -</i> 2582-3442		
<i>p-ISSN</i> - 2582-3558	Contents	
Original Articles		
Role of Centella Asiatica I Gnaneshvar Manivannan, Ray	E xtract in Scald Burns <i>r</i> i Kumar Chittoria, Neljo Thomas	9
Role of Onion Extract App Sagar Prakash, Ravi Kumar C	plication in Post-Varicella Scars hittoria, Neljo Thomas	15
Extraction of Anthocyanin Activities of its Phytochem Madhusudhan MC, S Lokesh	s from Seasonal Flowers and Biological icals	21
Guidelines for Authors		29

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Role of Centella Asiatica Extract in Scald Burns

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Abstract

In recent times, the research involving the use of medicinal plants in the modern medicine has increased all over the world. Many traditional medicinal plants have been incorporated into the modern medicine to aid in the process of wound healing. One such medicinal herb is Centella asiatica.Centella asiatica has been used in traditional medicine because of its ability to heal wounds and prevent scarring. Triterpenoid, saponins, the important constituents of Centella asiatica are mainly believed to be responsible for its therapeutic action. Apart from wound healing, the herb is recommended for the treatment of various skin conditions such as leprosy, lupus, varicose ulcers, eczema, psoriasis, diarrhea, fever, amenorrhea, diseases of the female genitourinary tract and also for relieving anxiety and improving cognition. This article is about the role of Centella asiatica extract in scald burns.

Keywords: Centella asiatica, Wound, Scald burns.

Introduction

Scald burns are one of the most common forms of non-accidental burn injuries. Scalds are a form of thermal burns resulting from contact of the skin with moist heat or heated fluids such as boiling water or steam. Usually in scald burns, due to the contact of the hot liquid with the epidermal layer of the skin, there is a superficial partial thickness loss of epidermis and part of the dermis. Hence most scalds are considered first or second degree burns. Healing of scald burns is a complicated process including inflammation, re-epithelialization, granulation, neovascularization and wound contraction. Although several preparations are available for the management of scald burns there is still a necessity of researching for efficacious medicine. Epithelialization is the natural act of healing dermal tissue resulting in minimal or no scarring.⁵ In most cases, scars occur if the depth reaches the dermis layer. When hypertrophic scar or keloid develops, it may induce itching, pain and even scar contracture.³ A lot of agents have been used to improve scars such as onion extract, resveratrol in grape's skin, curcumin and



Fig. 1: A 45 year old female with first degree scald burns on her lower abdomen and anteromedial aspect of both thighs.

centella. In this article we discuss about the role of Centella asiatica extract in scald burns. The role of Centella asiatica extract in scald burns is due to its anti oxidant and anti inflammatory and collagen remodelling property.⁶

Materials and Methods

This article is from the department of plastic surgery, JIPMER, Puducherry. After getting informed consent from the patient, clinical information and pictures are used in this article. A 45 year old female with no known comorbidities presented to the emergency department with alleged history sustaining scald burns by accidental spillage of hot water on her. Clinically her vitals were stable. She sustained first degree burns on her lower abdomen and anteromedial aspect of her both thighs (Figure 1). She was managed conservatively with daily cleaning and wound dressing with Cantella asiatica extract which is commercially available. Cantella asiatica extract was applied over the wound once in every 3 days for a period of 3 weeks (Figure 2 and 3)



Fig. 2: Application of Centella asiatica on the wound over the anteromedial aspect of left thigh

10



Fig. 3: Application of Centella asiatica on the wound over the lower abdomen

Results

the scald burns wound bed preparation was done with Cantella asiatica extract and was found to be useful. It allowed for skin grafting and allowed for good uptake of the same. (figure 4-6)

Discussion

The active compounds of Centella asiatica are pentacyclictriterpenes, including asiaticoside and madecassoside. In vitro studies demonstrated that asiaticoside decreases fibroblast proliferation in a dose-related manner and reduces the expression of both TGF- β I and TGF- β II at the level of transcription and translation.¹⁰ Asiaticoside also slows down scar formation by increasing the activity process of SMAD 7 which is a negative regulator of TGF-β signalling.¹¹ The other active composition, madecassoside acts by inhibiting the migration of fibroblasts.¹² Both active chemical substances promote C.asiatica to induce fibroblast proliferation and collagen synthesis. It involves the improvement of the tensile strength of newly formed skin and maturation of the scar by the







Fig. 4,5,6: Post skin grafting after using Centella for wound bed preparation

production of type I collagen.⁷ It's advantages include that it has low adherence to the wound bed. It does not have skin irritating potential and has no effects on serum biochemical profile when applied dermally. There is limited or no scar formation.^{8,9} However, it cannot be used in conditions like damage to skin caused by radiation therapy. It is safe when used for up to 10 weeks. It might cause itchiness and redness if used for longer period. Contact dermatitis^{13,14} can occur sometimes. Prolonged usage has shown cause some damage to the liver. This is especially seen in people who already have a compromised liver function. Such people should avoid using Centella asiatica as it has the propensity to further impair liver function.

Conclusion

Centella asiatica enhance wound bed preparation in patients with scald burns. Never the less, more large scale randomised control trials are needed ascertain the effects of C. asiatica extract on wound healing in patients with scald burns and its different parameters for use in clinical practice.

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Role of Onion Extract Application in Post-Varicella Scars

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Abstract

The efficacy of onion extract application in post varicella patients to reduce the hyperpigmentation caused due to the lesions secondary to the disease. Although the effectiveness and the process by which it reduces pigmentation is not established and evidence is inadequate, in our study, onion extract was applied a subject with post varicella scars and we evaluated the efficacy.

Keywords: Onion extract, Varicella scars, Scar reduction, Varicella zoster.

Introduction

Chickenpox or varicella is a contagious disease caused by the varicella-zoster virus (VZV). The virus is responsible for chickenpox (usually primary infection in non-immune hosts) and herpes zoster or shingles (following reactivation of latent infection). Chickenpox results in a skin rash that forms small itchy blisters which scab over. Varicella rash commonly evolves into permanent depressed scars, leaving life-long cosmetic issues for patients. Although there are a lot of reviews on depressed scars, the viral aetiology and the unique scar morphology of post varicella scar discriminate it from other depressed scars. Therefore, it is required to assess the efficacy of scar removal modalities on these scars, specifically. Yet, despite the prevalence, there is no comprehensive review on chickenpox scars' treatment, particularly. Evidence foronion extract usage and the process by which it reduces pigmentation and scarring in varicella-zoster is inadequate. The aim of the study is to evaluate the effectiveness of onion extract in post varicella scars.

Materials and Methods

This study was conducted in a tertiary care centre in the department of plastic surgery after getting the departmental ethical committee approval. Informed consent was obtained for examination and clinical photography. The subject was 23



Fig. 1: Pre intervention with varicella scars

years old male with history of varicella infection 2 weeks back following which he started noticing scars which were pigmented depressed scars with Vancouver Scar Scale (VSS) 6 (figure1). The patient presented to our OPD for scar management and was given onion extract for topical application daily for 6 weeks.

Results

After application of onion extract over a period of 6 weeks, in our study, we were able to successfully reduce the VSS and subjective improvement was present.(figure 2)

No adverse local or systemic effect was noted with use of onion extract.

Discussion

Varicella or chickenpox is caused by the varicellazoster virus (VZV), a herpesvirus with wide epidemiology. Post-primary infection, the virus is known to show a dormant residence in the body.¹ Spread of infection is via inhalation of infected aerosolized droplets. The virus can spread rapidly and has a high secondary attack rate. The initial infection is in the mucosa of the upper airways. After 2-6 days, the virus enters the circulation and another bout of viremia occurs in 10-12 days. At this time the characteristic vesicle appears. IgA, IgM, and IgG antibodies are produced but it is the IgG antibodies that confer lifelong immunity. After the primary infection, varicella localized to sensory nerves and may reactivate later to produce shingles.¹

Atrophic scars like PVS are very challenging to treat. Therefore, many techniques have been suggested to enhance the efficacy of treatment. Various methods are available for scar reduction post varicella infection. Among the various methods tried, an extract of Allium cepa, or onion, is one of the newer methods explored.² Although no extensive study is there in the literature to support the use of onion extract to reduce scar formation, some studies have found to have Quercetin, the active ingredient, which has been shown in invitro studies to decrease fibroblast proliferation, inflammation, extracellular matrix deposition, and



Fig. 2: Post onion extract application.

stabilized mast cells.3,4,5

Conclusion

We have found that onion extract has been very useful in management of post varicella scars but requires large scale randomized trials for large scale application to explore the potential of onion extract in the role of scar reduction.

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Extraction of Anthocyanins from Seasonal Flowers and Biological Activities of its Phytochemicals

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Abstract

Anthocyanins are water soluble vacuolar pigments that, depending on their pH, may appear red, purple, or blue found in plants. In the current study anthocyanin colors were extracted from locally available flowers of the *Pyrostegia venusta, Butea monosperma, Cassia auriculata, Tecoma stans and Woodfordia fruticosa* using acidified methanol as a solvent. Obtained extracts were evaluated for their phytochemicals and assessed for its bioactivity such as antimicrobial (*E coli, Staphylococcus aeries and Bacillus subtilis*) and antioxidant properties. This flower extracts has shown considerably significant activities along with their coloring property. The results show the potential usage of these natural colorants in various coloring applications including food coloration, alternative to synthetic colors. The study also explores the utilization of easily available sources and value addition to waste products.

Key words: Anthocyanin, Natural colorant, Medicinal plants, Antioxidants, Antimicrobials

Introduction

Colour is one of most important properties of food and beverages and is basis for their recognition and acceptability. Normally, food colours are due to naturally occurring pigments, but synthetic colorants are often added to confer the desired colour to the final product. Although synthetic colour had been favoured over the past 100 years, their use has been reduced due their harmful health effects in the past four decades. Consumer attention have turned towards naturally derived colours as a viable alternative to synthetic ones leads several avenues in search of pigment from various natural sources. Several natural food colors such as anthocyanins, betalains, chlorophylls, phycocyanins etc. were extracted from fruits, vegetables and other natural sources.¹⁻³

Anthocyanins are extracted from a wide variety of sources such as fruits (grapes, red 22

raspberry, cranberries),^{4,5} Vegetables (red cabbage, red radish, sweet potato)6,7 and petals of some flowers.^{8,9} Anthocyanin are considered as potential replacements for synthetic counterparts due to their bright attractive colour shades and ease of solubility that allows their incorporation into aqueous food system along with their known health benefits.¹⁰ Anthocyanins pigments extracted from flowers, fruits, and vegetables are traditionally used as dye and food colorant. Besides being used as natural colorants, some of the anthocyaninrich flowers and fruits have been traditionally used as medicine to treat various diseases. Plant anthocyanins have been widely studied for their medicinal values and known for anti-diabetic, anti-cancer, anti-inflammatory, anti-microbial, and anti-obesity effects and in addition to prevention of cardiovascular diseases (CVDs).11-16

Anthocyanins are the phenolic subclasses in the form of glycoside known as the aglycone. Anthocyanins are grouped into 3-hydroxyanthocyanidins, 3-deoxyanthocyanidins, and O-methylated anthocyanins. The most common types of anthocyanins are cyanidin, delphinidin, pelargonidin, peonidin, petunidin, and malvidin. Acylated anthocyanins are also detected in plants besides the typical anthocyanins.¹⁷

In the present study, the intense colored (Yellow, Pink and Red) flowers are used as an inexpensive sources of anthocyanins with potential to be used as natural, innocuous, and health beneficial colorants. The study involves isolation of anthocyanins from flowers of four selected plants *Pyrostegia venusta,Tacoma stans, Cassia arucilata, Woodfordia fruticosa andIpomoea cornea* and screening of biological activities. There are several scientific evidences with reference to these plants which could be potentially exploited for the extraction of various bioactives besides food colorants from these plants.

Material and Methods

Sample Collection

The flowers of the plants *Pyrostegia venusta, Tacoma stans, Cassia arucilata, Woodfordia fruticosa and lpomoea* cornea were collected from the nearby locality in and around Manasagangothri campus in Mysore, India. The flowers were cleaned, dried under shade and stored for further use.

Anthocyanin Extraction

The pigment from the flowers was extracted in acidified 50% methanol using a food processor. The solid to liquid content was maintained at 1:2 ratios.

The extracted pigment was filtered using cheese cloth to remove the fibrous particles and then it was centrifuged at 10,000 rpm for about 10 min to remove the tiny suspended particles. The obtained supernatant (anthocyanin extract) was stored at 4° C.

Anthocyanin Estimation

The pH differential method was used for quantitative determination of anthocyanins as reported by Guisti (2003).¹⁸ Each of two 0.2 ml aliquots was diluted with 2.8 ml of pH 1.0 buffer (125 ml of 0.2 N KCl and 385 ml of 0.2 N HCl) or pH 4.5 buffer (400 ml of 1 N sodium acetate, 240 ml of 1 N HCl and 360 ml distilled water) solutions, respectively. The samples were incubated for 30 minutes in dark and the absorbance was measured at 510 nm using UV / VIS spectrophotometer. Total anthocyanin pigments were determined from absorbance in pH 1.0 buffer, while monomeric anthocyanins were determined from the differences between absorbance in pH 1.0 and 4.5 buffers.

The anthocyanin content in all the extract was calculated using the following equation

Anthocyanin pigment (mg/100ml)

$$= \frac{A \times Mw \times DF \times 100}{\varepsilon \times L}$$

where A= A_{510} (pH 1.0)– A_{510} (pH 4.5), Mw is the molecular weight of anthocyanin (433.2 g/mol), DF is the dilution factor, ε is the extinction coefficient (31,600 L/cm mol) and Lis the path length (1 cm).

Phytochemical Analysis

Qualitative estimations of phytochemicals were carried out according to standard procedures reported in the literature.¹⁹

Test for Carbohydrates

To 2ml of plant extract, 1ml of Molisch's reagent (α -naphthol dissolved in ethanol) and few drops of concentrated sulphuric acid were added. Presence of purple or reddish color indicates the presence of carbohydrates.

Test for Tannins

To 1ml of plant extract, 2ml of 5% ferric chloride (5% w/v solution of ferric chloride prepared in 90% alcohol) was added. Appearance of a dark green or deep blue colour indicated the presence of tannins.

Test for Flavonoids

To 2ml of the extract, a few drops of 10% ferric chloride solution were added. A green blue or violet colouration indicated the presence of Flavonoids.

Test for Alkaloids

To 2ml of plant extract, 2ml of concentrated hydrochloric acid was added. Then few drops of Mayer's reagent were added. Presence of green color or white precipitate indicates the presence of alkaloids.

Test for Quinones

To 1ml of extract, 1 ml of concentrated sulphuric acid was added. Formation of red color indicates presence of quinones.

Test for Phenols

To 1 ml of the extract, 2 ml of distilled water followed by few drops of 10% ferric chloride was added. Formation of blue or green color indicates presence of phenols.

Test for Terpenoids (Salkowski Test)

To 0.5ml of extract, 2ml of chloroform was added, followed by a further addition of 3ml of conc. H2SO4 to form a layer. A reddish brown colouration of the interface indicated the presence of terpenoids.

Test for Cardiac Glycosides (Keller-Killiani Test)

To 0.5 ml of extract, 2 ml of glacial acetic acid and few drops of 5% ferric chloride were added. This was underplayed with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of cardiac glycoside.

Test for Proteins

The extract was taken with 2 ml of water and 0.5 ml of concentrated HNO_3 was added to it. Yellow colour is obtained if proteins are present.

Test for Triterpenoids

To 1.5ml of extract, 1ml of Libemann –Buchard Reagent (aectic anhydride concentrated sulphuric acid) was added. Formation of blue green color indicates presence of triterpenoids.

Test for Coumarins

To 1 ml of extract, 1ml of 10% NaOH was added. Formation of yellow color indicates presence of coumarins.

Test for Anthraquinones

To 1ml of plant extract few drops of 10% ammonia solution was added, appearance pink color precipitate indicates the presence of anthraquinones.

Test for Saponins

To 2 ml of plant extract, 2 ml of distilled water was added and shaken in a graduated cylinder for 15 minutes lengthwise. Formation of 1 cm layer of foam indicates the presence of saponins. The persisted frothing on warming was taken as an evidence for the presence of saponins.

Test for Sugars (Fehling's test for Free Reducing Sugar)

About 0.5g of each extract was dissolved in distilled water and filtered. The filtrate was heated with 5ml of equal volumes of Fehling's solution A and B. Formation of a red precipitate of cuprous oxide was an indication of the presence of reducing sugars.

Test of Steriods (Salkowski's test).

To 1 ml of the extract was added to 1 ml of chloroform in a test tube. Concentrated sulphuric acid was carefully added from the sides of the test tube, to form a layer. A reddish brown colour at the interphase indicated the presence of steroids.

Test for Glycosides

To 2 ml of plant extract, 3 ml of choloroform and 10% ammonia solution was added. Formation of pink color indicates presence of glycosides.

Phlobatannins

To 1ml of plant extract few drops of 2% HCL was added appearance of red color precipitate indicates the presence of phlobatannins.

Antibacterial Activity

Antibacterial activity of the extracts was carried out by agar well diffusion assay reported by Gonelimali et al.(2018)²⁰ with some modifications. Petri dishes were prepared by pouring 20 ml of sterilized nutrient agar media under aseptic condition and allowed to solidify. After solidification of the media, 100 µl of standardized test microbial inoculum of *S. aureus, B. subtilisand E. coli* were inoculated and spread uniformly using sterile spreader. 6 mm diameter agar is drawn from plate to form a well using sterile cork borer. Antibiotic Streptomycin was used as positive control. After keeping at 4 °C for 4 hours for the diffusion of anti-bacterial metabolites, thereafter plates were incubated at 37 °C for 24 h. The diameter of the inhibition zone around the well is measured in millimeter (mm) and the average of three repeated agar discs were taken to assess the strength of antibacterial activity.

Antioxidant Assay

DPPH Scavenging Assay

The free radical scavenging capacity of the extracts was determined by DPPH method reported by Williams et al. $(1995)^{21}$ with minor modifications. The DPPH radical solution (300µM) was prepared in ethanol kept in dark. 0.2 ml of Different concentrations of test samples 2.8 ml of DPPH was added. The tubes were incubated for 30 min at room temperature and the absorbance was recorded at 517 nm. Ascorbic acid (AA) was used as positive control. The free radical scavenging activity of samples was expressed in percentage and each sample was analyzed in triplicate. The free radical scavenging activity was calculated by using the following equation:

Scavenging activity (%) = $\frac{[Aa-(Ab-Ac)]}{Aa}x100$

where Aa is the absorbance of the control solution of DPPH (without anthocyanin extract), Ab is the absorbance of the mixture containing anthocyanin extract as well as DPPH, and Ac is the absorbance of the blank solution without DPPH.

Results and Discussion

Anthocyanin estimation

Estimation of anthocyanin is carried out using pH



Fig. 1: Concentration of anthocyanins extracted from flowers



Fig. 2: Radical scavenging activity (%) of flower extracts (anthocyanin)

differential method as per the standard protocol and calculated the concentration employing the equation given. The obtained results were presented in Fig. 1. Highest concentration of anthocyanin 48.5 mg/100ml is observed in flower extract of *Woodfordia fruticosa* and lowest concentration of anthocyanin 12.3 mg/100ml is with flower extracts of *Tecoma tans*. Similarly, other extracts were found to be *Pyrostegia venusta* is 30.2 mg/100ml, *Butea monosperma* is 38.5mg/100ml and *Cassia auriculata* is 28.5mg/100ml.

Phytochemical Tests (qualitative analysis)

The phytochemical analysis of methanol extracts of flowers *Pyrostegia venusta*, *Butea monosperma*, *Cassia auriculata*, *Tecoma stans* and *Woodfordia fruticosa* were carried out employing standard protocols. The results are tabulated in Table 1. The presence of the Tannins, Saphonins, Flavonoids, Quinones, cardiac glycosids, phenols were observed in all extracts of flowers. The extracts of *Butea monosprma* and *cassia auriculata* has shown the presence of steroids. The presences of the phytochemicals are the indications of their bioactivities they have.

Antimicrobial Activity

Antibacterial activity was carried out using disc diffusion method. Three different concentrations of the compounds (10, 20, 30 μ l) along with standard Streptomycin (1mg/ml, 10 μ l sample) was used as a positive control. These plates were incubated for 24 hrs at 37 °C. Zone of inhibition was recorded in millimeters (mm). Observed results were tabulated in Table 2.

Inhibition of bacterial growth around the sample was observed in all the samples studied which shows the antimicrobial potential of the anthocyanin extracts. The best antimicrobial activity was observed at Woodfordia fruticosaflower extract which has shown highest inhibition against Staphylococcus aureus, zone of inhibition observed was around 2.6mm. The best antimicrobial activity of Pyrostegia venustaflower extract was against Staphylococcus aureus (2.2mm). The best activity of Buteamono sperma flower extract was against Bacillus subtilis (2.2mm). The best activity of Cassia auriculataflower extract was against Staphylococcus aureus (1.6mm). The best activity of Teccoma stans flower extract was against Bacillus subtilis (1.6mm). It was observed that zone of inhibition

S1. no.	Name of the flower	Pyrostegia venusta	Butea monosperma	Cassia auriculata	Tecoma stans	Woodfordia fruticosa
1	Carbohydrate	-	-	-	-	-
2	Tannins	+	+	+	+	+
3	Saponins	+	+	+	+	+
4	Flavonoids	+	+	+	-	+
5	Alkaloids	-	-	-	-	-
6	Quinones	+	+	+	+	+
7	Cardiac glycosides	+	+	+	+	+
8	Trpinoids	-	-	-	-	-
9	Tritrpinoids	-	-	-	-	-
10	Phenols	+	+	+	+	+
11	Coumarin	+	+	+	+	+
12	Steroids	-	+	+	-	-
13	Phytosteroids	-	-	-	-	-
14	Protein	-	-	-	-	-
15	Sugar	-	-	-	+	-
16	Anthoquinones	-	-	+	-	-

Table 1: Phytochemical analysis of anthocyanin extract

Table 2: Antimicrobial activity (Zone of inhibition)

Flowers of different	*Zone of inhibition (mm) of bacterial colonies						
plants used	Concentration	Escherichia coli	Staphylococcus aureus	Bacillus subtilis			
Pyrostegia venusta	Control	2.0	2.0	2.0			
	10µL	0.8	1.0	0.8			
	$20 \mu L$	1.0	1.8	1.4			
	30µL	1.6	2.2	1.9			
Butea monosperma	Control	2.0	2.1	2.0			
	10µL	1.0	0.8	1.1			
	$20\mu L$	1.4	1.5	1.6			
	30µL	1.6	2.0	2.2			
Cassia auriculata	Control	2.0	2.0	1.9			
	10µL	0.6	0.7	0.8			
	20µL	1.0	1.0	1.0			
	30µL	1.4	1.6	1.5			
Teccoma stans	Control	2.1	2.0	2.0			
	10µL	0.5	0.5	0.6			
	20µL	0.6	0.7	1.0			
	30µL	0.8	1.0	1.6			
Woodfordia fruticosa	Control	2.0	2.0	2.1			
	10µL	1.0	1.2	1.2			
	20µL	1.6	1.9	1.7			
	30µL	2.0	2.6	2.4			

.Data based on the average of triplicates in vitro

was increased with increased content. The antimicrobial activities were varied with respect to the extract and the organism studied. The activity may be due to the content and also depending on the phytoconstituents of each extracts.

Antioxidant Activity

DPPH Free Radical Scavenging Activity

The free radical scavenging activity of flowers of *Pyrostegia venusta, Butea monosperma, Cassia auriculata, Tecoma Stans and Woodfordia fruticosa* was carried out using 2,2-diphenyl-picrylhydarzyl whose free radical were to be scavenged by the sample extract using the ascorbic equivalence standard. All the samples tested were shown significantly good radical scavenging activity. However the % scavenging activity varies with the samples (Fig. 2).

The best radical scavenging activity was observed in *Woodfordia fruticosa extrct* compared to other flower extracts which exhibits 93.45% antioxidant activityfollowed by *Butea monosperma* (91.0%) and lowest was with *Pyrostegia venusta* extract (52.61%). The results indicate the variation of % antioxidant activity may be due to the variation in anthocyanin concentrations and also presence of the variousphy to chemicalsin different extracts.

Because of the growing awareness on importance of naturally derived products over synthetic ones, several attempts have been made on processing of natural colors from various natural sources. The current study is also demonstrates the possibility of isolation of natural pigments from easily available flowers from selected plants which are also known for some medicinal properties. Freely available flower sources such as Pyrostegia venusta, Butea monosperma, Cassia auriculata, Tecoma stans and Woodfordia fruticosawere rich in anthocyanin and could be used as good sources of producing different shades of colours. Further, the study also explores the usage of these extracts not only to color, but also its other health benefits such as potent antioxidant and antimicrobial properties. Natural antioxidants have demonstrated beneficial effects in maintenance of health, management of age related diseases, ameliorating due to the harmful effects of toxic agents both chemical and physical. There are several evidences available to understanding of effects of natural antioxidants.

Conclusion

Increasing demand for natural food colorant in comparison to synthetic one is mainly due to the health promoting properties besides its coloring nature. The market for natural food colours is estimated to increase by approximately 10% annually leading for the exploration of newer sources for these products from natural origin. In this view this anthocyanin pigments were extracted from naturally available sources which are rich in pigments and the studies revealed the significant levels of biological activities of these extracts.

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Kidd EAM, editors. Dental caries: The disease and its clinical management. Oxford: Blackwell Munksgaard; 2003. pp 7-27.

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Reference from electronic media

[9] National Statistics Online – Trends in suicide by method in England and Wales, 1979–2001. www. statistics.gov.uk/downloads/theme_health/HSQ 20.pdf (accessed Jan 24, 2005): 7–18. Only verified references against the original documents should be cited. Authors are responsible for the accuracy and completeness of their references and for correct text citation. The number of reference should be kept limited to 20 in case of major communications and 10 for short communications.

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