EVALUATION AND ANALYSIS OF ANNATTO EXTRACT FOR TOCOTRIENOLS

A Dissertation submitted by

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(REG NO. VM20FPT013)

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Under the guidance of

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CERTIFICATE

This is to certify that the investigation entitled "**Evaluation and analysis of annatto extract for tocotrienols**", submitted by **Ms. SREELAKSHMI V.S**., (Reg no: **VM20FPT013**) during February 2022 – July 2022, in the partial fulfilment for award of the degree of **Master of Vocational Studies** in **Food Processing Technology**, Kerala, is the result of investigation carried out by her in department of **Spices & Flavour Sciences**, at CSIR- Central Food Technology Research Institute, Mysore, under the guidance of **Dr. SHIVAKUMAR L.**

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DECLARATION

I **Sreelakshmi.V.S.** (Reg no:**VM20FPT013**), hereby declare that the project work entitled "**Evaluation and analysis of annatto extract for tocotrienols**", submitted to Mahatma Gandhi University, Kottayam in partial fulfilment of the requirements for the award of the degree of **Master of Vocational Studies** in **Food Processing Technology**, an authentic record of the original research work carried out by me during the period from February 25 to July 17, 2022 under the supervision and guidance of **Dr. Shivakumar L**., Scientist, Department of Spice and Flavour Science, CSIR-Central Food Technology Research Institute, Mysore. I also declare that this project has not been submitted to any other universities or institutions for the award of any degree.

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Place: Mysore

Date:

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ABBREVATIONS

α	Alpha
β	Beta
γ	Gamma
δ	Delta
BALB/c	Albino immunodeficient laboratory bred house mouse
CC	Column Chromatography
CEC	Capillary Electrochromatography
CO_2	Carbon dioxide
GC	Gas Chromatography
GC-MS	Gas Chromatography- Mass Spectroscopy
DAD	Diode Array Detection
DCM	Dichloromethane
DMBA	Dimethylbenz[a]anthracene
ELSD	Evaporative Light Scattering Detector
g	gram
GC	Gas Chromatography
GC-MS	Gas Chromatography- Mass Spectroscopy
Hep3B cells	liver cancer cell line
HPLC	High Pressure Liquid Chromatography
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme-A
HT, HT-4	Hippocampal neuronal cells
Kg	kilogram
KOH	Potassium hydroxide

MDA-MB	M.D. Anderson- Metastatic Breast
Mpa	mega Pascal
MS	Mass Spectroscopy
m	meter
mg	milligram
ml	milliliter
mm	millimeter
min	minutes
nm	nanometer
NaOH	sodium hydroxide
NMR	Nuclear Magnetic Resonance
NP	Normal Phase
PC	Prostate cancer
PDA	Photo Diode Array
psi	one pound per square inch
RP	Reverse Phase
SFE	Supercritical Fluid Extraction
T	tocopherol
T3	tocotrienol
TLC	Thin Layer Chromatography
TRF	Tocotrienol Rich Fraction
UV	Ultraviolet
°C	Degree Celsius
%	percentage

ABSTRACT

The annatto seeds reported to be a rich source for δ-tocotrienols, which are known potential biomolecules having anti-cholesterolemic and anti-cancer properties. Under this context, the present study proposed for the isolation of tocotrienols by preparing the hexane extract by Soxhlet extraction followed by silica gel column chromatography gave the tocotrienols rich conserve having two major components with RT 2.06 and 2.29 respectively as analysed by HPLC under standardized conditions. The HPLC analysis with PDA detection indicated the λmax value of 297 nm which corresponds to the reported value of δ-tocotrienol. This got eluted with the RT of 2.06 and showed the relative percent of 62.395 which is considerably a higher proportion. Further identification was carried out by doing a facile acetylation using pyridine and acetic anhydride followed by purification by column chromatography and subsequently by GC-MS analysis. GC-MS profile indicates 0.336% of δ-tocotrienol acetate wherein the major component was found to be geranylgeraniol (83.667%) with RT 19.160. This may be attributed to degraded product of δ-tocotrienol and rearranged to geranylgeraniol. Thus, the preliminary work carried out on isolation of tocotrienols from annatto seed extract showed 62.395 % of δ-tocotrienol by HPLC, which is considered to be a significant proportion in the extract which is further validated by derivatization and GC-MS analysis.

INTRODUCTION

ANNATTO

Bixa orellana L. is a species belonging to the Angiospermae subdivision, Dicotiledoneae class, Parietales order, Cristianeae suborder, Bixaceae family and Bixa genus (Ramalho et al., 1987). It is a small tree, usually less than 6 m, reaching up to 8 m, with a stem base diameter of 15-20 cm, wide copse and abundantly branched (Cruz et al., 2008). The fruits are ovoid and flattened capsules, oval, hemispherical ellipsoid or conical, containing numerous seeds, surrounded by a reddish pulp. The capsules open in two equal parts and depending on the plant variety, the capsules might be dark brown, reddish, green, or pale yellow (Costa et al., 2008). They feature an average of 54 pigment-containing seeds and are heavily covered in flexible spines. Fruiting happens all year round, but it increases significantly in the winter. Late summer and early fall are when the fruits begin to ripen. In the second year, fruiting begins, and by the third year, the products are ready for sale. Each fruit may have a large number of seeds. They are 34 mm long, angular, and covered in a yellowish-red resinous substance that, as it ages, turns dry, hard, and dark (Fig. 1). Bixin, which accounts for more than 80% of all carotenoids, is the pigment found in higher concentration in the seeds (Stringheta and Silva, 2008; Costa et al., 2008). The common names for *Bixa orellana* L. are Lipstick tree, Achiote etc.

Fig. 1. **Annatto seeds (**www.sciencedirect.com**)**

CHEMICAL COMPOSITION OF ANNATTO PIGMENT

Natural carotenoid colourants from the pericarp of the seeds of the tropical annatto tree *(Bixa orellana* L.) are used to make annatto food colours. A highly coloured resin made mostly of the carotenoid bixin is present on the seed's surface. A unique procedure is used to extract the bixin from the seed, yielding the current form for usage in the creation of various food products. The earliest colour known to man, annatto is one of the most widely used food colourings in the world. In addition to colouring cheese, butter, and other dairy goods, it is also used in soft beverages, sugar confections, margarine, ice cream, and fish products. Bixin [**6′-methyl hydrogen (9′Z)-cis-6, 6′-diapocarotene-6, 6′-dioate**] (Shahid-ul-Islam et al., 2015) is a diapocarotenoid, mainly present in annatto seeds, accounting for at least 80% of total carotenoids present in the seed and is the fat soluble pigment present in the annatto seeds. (Shahid-ul-Islam et al., 2014). While the pigment called norbixin **[(2E,4E,6E,8E,10E,12E,14E,16E,18E)-4,8,13,17-tetramethylicosa-2,4,6,8,10,12,14,16,18-nonaenedioic acid]** and its salt are the major pigments for water-soluble preparation of annatto (Stringheta et al., 2008). Bixin is the methyl ester of dicarboxylic acid norbixin (Scotter, 2009). It is soluble in concentrated alkaline solutions, where it undergoes saponification, forming a norbixin salt. This salt when in acidic media forms dicarboxylic acid norbixin (Silva and Stringheta, 2008).

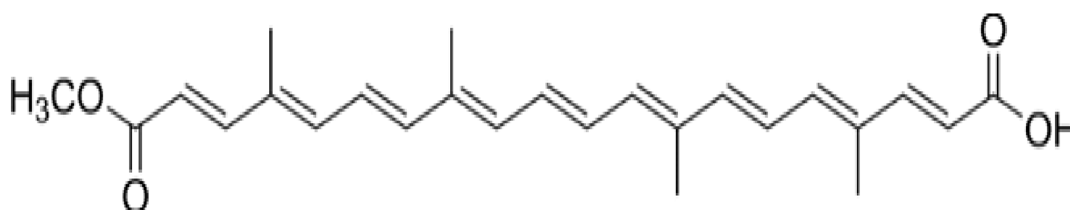

Fig. 2. **Bixin** (www.researchgate.com)

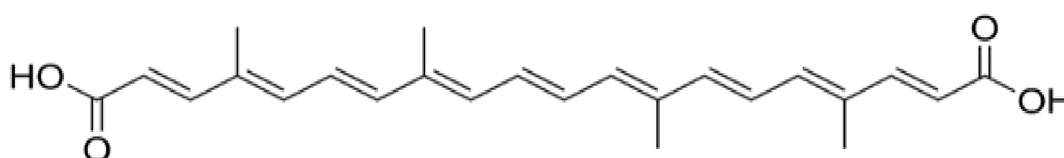

Fig. 3. **Norbixin** (www.researchgate.com)

EXTRACTION OF ANNATTO SEEDS

Three main commercial processes are used to extract the pigment from dried annatto seeds. These are (i) extraction into oil, (ii) extraction with solvents and (iii) extraction into aqueous alkali (Preston and Rickard 1980).

Extraction into oil

Oil soluble bixin: Annatto seeds, immersed in vegetable oil at a temperature not exceeding 70 °C are mechanically abraded to remove the pericarp from the waste seed by process known as "raspelling". The slurry of the pigment is heated under vacuum at temperature below 130 °C. The solution is filtered to remove insoluble materials. The main carotenoids include trans-bixin, a yellow thermal degradation product and cis-bixin (Preston and Rickard 1980).

Suspension in oil: The extract of the pericarp in oil after 'raspelling' is centrifuged and the finer particles are blended with oil to give a suspension in oil without thermal treatment (Preston and Rickard 1980).

Extraction by mono and triglycerides, free fatty acids and propylene glycol of food grade is also employed for the extraction of the oil soluble bixin from the annatto seeds.

Extraction into solvents

Bixin is a non-polar chemical with a strong affinity for polar solvents like acetone, ethyl acetate, methanol, and ethanol. According to (Cardarell et al.,), the most effective solvent for extracting bixin from annatto seed is ethyl acetate but recently other solvents and techniques have been introduced to extract bixin. The annatto pigment is extracted into a suitable solvent and the extract is washed with another solvent in which the pigment is virtually insoluble, to remove unwanted impurities and odours, for production of annatto crystalline dye with concentration of bixin (Preston and Rickard 1980). Other solvents used for extraction are acetone, dichloromethane, dichloroethane, ethanol, light petroleum and propan-2-ol etc.

Oil extraction and solvent extraction are employed for the extraction of the oil soluble bixin fraction, whereas to extract the water soluble fraction norbixin alkali extraction is employed.

Extraction into aqueous alkali for production of water soluble annatto

From seed: The pigment of the annatto seed is extract by agitation in aqueous alkali at temperatures not exceeding 70°C. It will provide the aqueous solution with sodium or potassium salts of norbixin, probably in the cis and trans forms. An approach for producing spray-dried orange red pigment powder from annatto was described by (Park et al., 1990). The seeds were exposed to a solution of sodium hydroxide (1-2%) in ratio 1:1 to 1:3, for 10–20 minutes at room temperature. Subsequently the extract was spray dried.

From solvent extracted bixin: Bixin, derived from solvent extraction of the pericarp by one or more stages, followed by removal of the solvent is hydrolysed with aqueous alkali to yield the sodium or potassium salts of norbixin (Preston and Rickard 1980). Using alkaline propylene glycol to extract annatto seeds, (Kocher, 1958) produced the alkaline salts of cis- and trans-norbixin. A study by (Dendy, 1966a) reported an alkaline extraction method followed by acid treatment.

Supercritical carbon dioxide fluid extraction

Using supercritical carbon dioxide at 310 bar and 50°C as a solvent, certain purity and degradation issues were resolved (Chao et al., 1991). Supercritical carbon dioxide was employed by (Degnan et al., 1991) to extract bixin from the seed coat and analyse the solubility of pure bixin at pressures and temperatures of 3000-7000 psi and 40-55°C, respectively. Pure bixin had a maximum solubility of 0.003 and annatto seed pigment had a maximum solubility of 0.26 mg/g of carbon dioxide. There was no thermal degradation during the extraction and the supercritical extraction method was found useful only for extraction of bixin and not norbixin from a mixture. The presence of soybean oil improved extraction efficiency and decreased the amount of carbon dioxide needed from 350 to 90 g to extract 2.7 mg of pigment. In a mix of static and dynamic modes of extraction, (Anderson et al., 1997) reported extracting trans-bixin utilising supercritical fluid extraction. In order to optimise the extraction conditions, the HPLC was used to measure the extraction efficiency of bixin. At 40°C and 60.62 Mpa, the scientists used carbon dioxide containing acetonitrile and 0.05 percent trifluoroacetic acid as a modifier to obtain 2.7 mg bixin per gram of annatto seed.

VITAMIN E

Vitamin E is a term that encompasses a group of potent, lipid-soluble, chain-breaking antioxidants. It is the generic term applied to a group of eight vitamins, tocopherols (α-, β-, γ- and δ-T) and tocotrienols (α-, β-, γ- and δ-T3), all of which have biological activity. Both structures are similar except that the tocotrienol structure has double bonds on the isoprenoid units. The main interest in vitamin E is its natural antioxidant capability; it is considered to be one of the most efficient biological antioxidants for breaking free radical chain reactions. Thus, it protects cytoplasmic membranes from oxidation and guards low-density lipoproteins from dangerous lipid peroxidation processes. In addition, its antioxidative capacity and its ability to act as a free radical scavenger can reduce the risk of cancer and delay the progression of precancerous lesions. Tocotrienols reduce plasma cholesterol levels, as well as those of other lipids and non-lipids related to risk factors for cardiovascular diseases. In the field of cancer chemotherapy, tocotrienols display better anti-tumour activity than α-tocopherol. In recent years, tocotrienols and tocopherols have been included as analytes in vitamin E determinations. Most vegetable oils and biological fluids contain varying amounts of tocopherols. Nevertheless, tocotrienols can be found in palm oil, coconut oil and cereal grains, such as wheat, rye, oats, barley and annatto seeds.

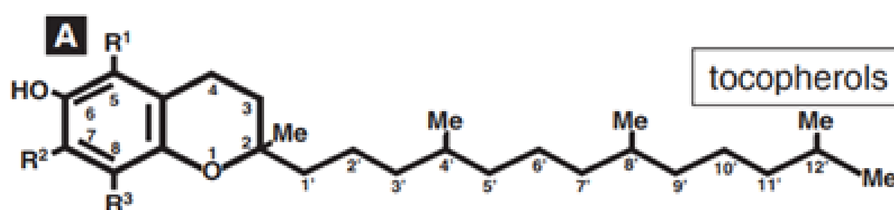

Fig. 4. **Tocopherol** (www.mdpi.com)

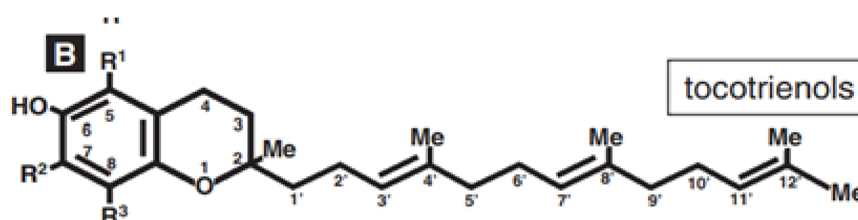

Fig. 5. **Tocotrienol** (www.mdpi.com)

AIM AND OBJECTIVES

Tocopherols and tocotrienols are the members of the Vitamin E family. Tocopherols has been studied vastly by researchers but the tocotrienols are less explored. But recent studies have proven that tocotrienols exhibit neuroprotective, anticancer and cholesterol lowering properties that are not often exhibited by tocopherols. And in recent studies annatto was identified as a rich resource of tocotrienols, surpassing two other known resources, palm oil and rice bran. This study aims the “**Extraction and analysis of annatto extract for tocotrienols**”

OBJECTIVES

- To extract the tocotrienols from annatto by Soxhlet extraction using a suitable solvent
- To separate the compounds by chromatography and characterization.

REVIEW OF LITERATURE

BIOLOGICAL FUNCTIONS OF TOCOTRIENOLS

All eight vitamin E tocols have equal antioxidant activity because of their near structural similarities. Nevertheless, research on the biological effects of vitamin E shows that its members have distinct biological properties that are frequently not shared by other family members. Tocotrienol performs a variety of tasks that tocopherol does not (Sen et al., 2006). For instance, tocotrienol in nanomolar quantities prevents inducible neurodegeneration in a special way by controlling particular cell death mediators (Khanna et al., 2003, 2006; Sen et al., 2000). Tocotrienol oral supplementation prevents stroke (Khanna et al., 2005b). The hepatic enzyme in charge of synthesising cholesterol, 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase, is inhibited by micromolar concentrations of tocotrienol (Pearce et al., 1992, 1994).

Administration of tocotrienol, not tocopherol, lowers oxidative protein damage and raises the average lifespan of *Caenorhabditis elegans* (Adachi and Ishii, 2000). It is believed that tocotrienols are more powerful antioxidants than tocopherol (Serbinova and Packer, 1994; Serbinova et al., 1991). Tocotrienol's unsaturated side chain makes it more effective at penetrating tissues with saturated fatty layers, like the brain and liver (Suzuki et al., 1993). Tocotrienols appear to be superior to tocopherol due to their greater dispersion in the fatty layers of the cell membrane, according to experimental study comparing the antioxidant and free-radical scavenging activities of the two substances (Suzuki et al., 1993). Additionally, the proliferation of human breast cancer cells is suppressed by tocotrienol but not tocopherol.

When tocotrienol is supplemented, it raises α-tocotrienol levels in the blood plasma to a peak that is more than an order of magnitude greater than what is necessary to shield neurons from a variety of neurotoxic assaults. The "minor" vitamin E compounds were almost entirely dismissed by biologists as subjects for basic and clinical research because to the specific vitamin activity of tocopherol, its abundance in the human body, and the equal efficiency of tocopherols as chain breaking antioxidants. Recent findings call for a thorough re-examination of this conventional thinking.

STRUCTURE OF TOCOTRIENOLS

According to (Fereidoon et al., 2016), tocotrienols are monophenols that come in four homologues (alpha, beta, delta, and gamma) that differ from one another in terms of number and location of methyl groups in their chemical structures. Tocotrienols have three double bonds at positions 3', 7' and 11' of the side chain. Only the β- and γ forms of tocopherols or tocotrienols can be called isomers, having the same formula but a different arrangement of atoms in the molecule.

Type	R1	R2	R3
α-tocotrienol	Me	Me	Me
β-tocotrienol	Me	H	Me
γ-tocotrienol	H	Me	Me
δ-tocotrienol	H	H	Me

Table 1. **Various homologs of tocotrienols**

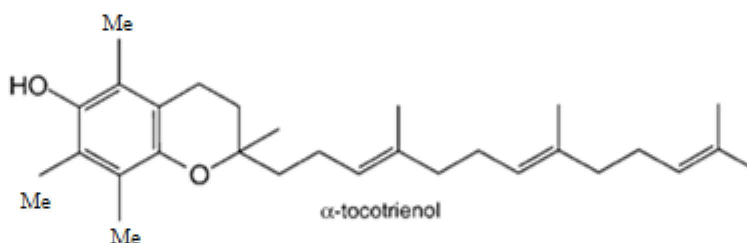

Fig. 6. **α- tocotrienol** (Int. J. Mol. Sci. 2016, 17, 1745)

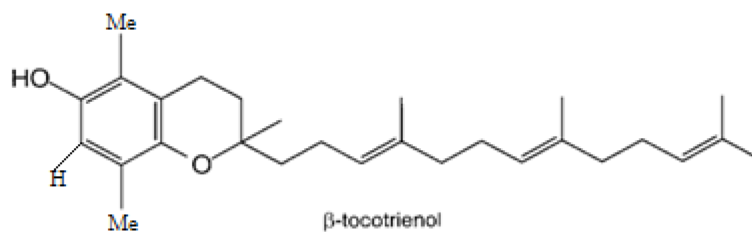

Fig. 7. **β- tocotrienol** (Int. J. Mol. Sci. 2016, 17, 1745)

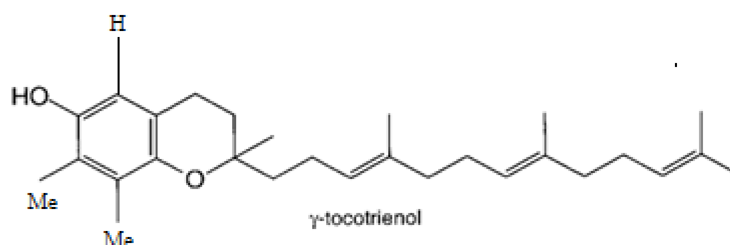

Fig. 8. **γ- tocotrienol** (Int. J. Mol. Sci. 2016, 17, 1745)

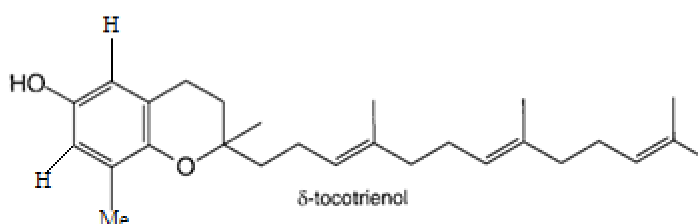

Fig. 9. **δ- tocotrienol** (Int. J. Mol. Sci. 2016, 17, 1745)

NATURAL SOURCES OF TOCOTRIENOLS

Both edible and inedible plant sources contain tocotrienol. A significant non-food natural source of tocotrienols is rubber latex (Chow and Draper, 1970), (Horvath et al., 2006), (Whittle et al., 1966). A significant early discovery demonstrating the special significance of tocotrienols in health and disease was the identification of tocotrienol as a cholesterogenesis inhibitory factor in barley (*Hordeum vulgare* L.) (Qureshi et al., 1986).

One of the most plentiful natural sources of tocotrienols is palm oil (Elson, 1992). In palm oil, vitamin E is distributed as 30% tocopherols and 70% tocotrienols (Sundram et al., 2003). Many West African nations are home to the oil palm (*Elaeis guineensis*), whose oil has long been used by the indigenous populace for both culinary and other uses. Large-scale plantations are mostly created in tropical areas of Asia, Africa, and Latin America with the intention of producing oil, which is derived from the fleshy mesocarp of the palm fruit, and endosperm or kernel oil (Solomons and Orozco, 2003). Because it includes 50% saturated fatty acids, 40% unsaturated fatty

acids, and 10% polyunsaturated fatty acids, palm oil differs from other plant and animal oils.. Palm oil has not been very well-liked due to its high saturated fat level. Some other foods and plants, including rice bran oil, coconut oil, wheat germ, and annatto, contain tocotrienols. Tocotrienol levels in palm oil and rice bran oil were thought to be particularly high (940 and 465 mg/kg, respectively). Oats, hazelnuts, maize, olive oil, Buckthorn berry, rye, flax seed, poppy seed, and sunflower oil are other sources of tocotrienols.

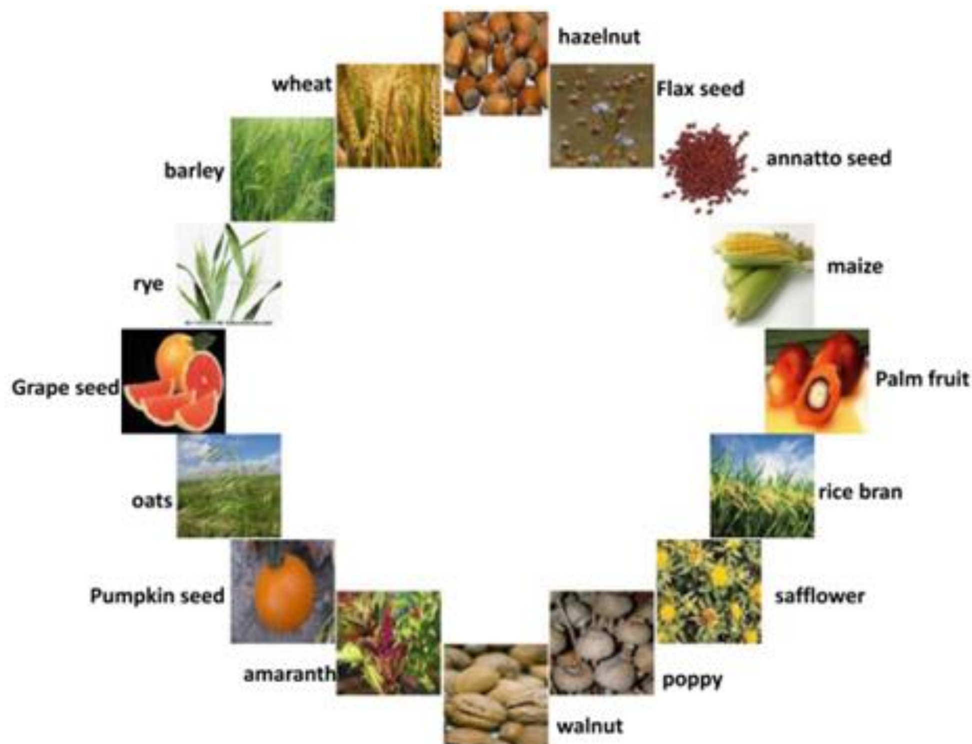

Fig. 10. **Natural sources of tocotrienols** (www.springer.com)

BIOLOGICAL FUNCTIONS OF TOCOTRIENOLS

Neurodegeneration

Neurodegeneration is largely attributed to glutamate poisoning (Schubert and Piasecki, 2001; Tan et al., 2001). In a 1999 study, all eight naturally occurring forms of vitamin E were compared side by side in a model of glutamate-induced neurodegeneration of HT neural cells. In later studies, it was discovered that

tocotrienol's neuroprotective function extends to primary cortical neurons as well as neural cell lines. This line of research finally led to a finding that ultimately proved to be the most effective action of any naturally occurring vitamin E on a reported concentration basis. Up until that point, only micromolar concentrations of vitamin E were used to examine its biological effects in vitro. The studies led to the first evidence that α-tocotrienol was the most potent neuroprotective form of vitamin E in glutamate-induced degeneration of HT-4 hippocampal neurons (Sen et al., 2000).

This study found that complete nueroprotection is provided by α-tocotrienol nanomolar quantities rather than α-tocopherol. Not only does tocotrienol have neuroprotective properties in response to glutamate challenge, but also in reaction to other insults such homocysteic acid, glutathione deprivation, and oxidative damage caused by linoleic acid (Khanna et al., 2006; Sen et al., 2000). It is now clear that tocotrienol, which has antioxidant properties, protects brain cells at micromolar doses. Tocotrienol, however, controls particular neurodegenerative signalling mechanisms at nanomolar doses.

Anti-cancer

Potent anticancer properties exist for both pure and mixed isoprenoids (Mo and Elson, 1999). Tocopherols are not isoprenoids, although tocotrienols are. Although α -tocotrienol has emerged to be the most potent isoform in case of neuroprotection (Khanna et al., 2005b, 2006; Sen et al., 2004, 2006), there seems that γ- and δ-tocotrienols are the most potent anticancer isoform of all natural existing tocotrienols. The effects of intraperitoneally injected α- and γ-tocotrienols, as well as that of α-tocopherol, have been examined. Invasive mammary carcinoma, Ehrlich carcinoma, and Sarcoma 180 were all successfully treated with both tocotrienols. In mice with Meth A fibrosarcoma, γ- tocotrienol had a marginally life-extending impact (Komiyama et al., 1989). Tocopherol was less effective than tocotrienols in this experiment.

The antitumor activity of γ-tocotrienol was seemed to be higher than that of α-tocotrienol. When exposed to tocotrienols for 72 hours in vitro, human and mouse tumour cells displayed growth inhibition as opposed to tocopherol (Komiyama et al., 1989).

Tocotrienol's anticancer properties are unrelated to its antioxidant or redox properties. It is clear that the phytyl side chain in tocotrienol has certain antioxidant properties that guard against carcinogenesis, even if the phenolic antioxidant group may not be directly responsible for this property's anticancer effects.

Breast cancer

The effectiveness of tocotrienols has been investigated most thoroughly in cell culture and mouse in vivo models for breast cancer. In a rat mammary tumour model created using chemicals, the chemopreventive effects of tocopherol and tocotrienol have been compared side by side. The tocotrienol group was the only one to exhibit improved tumour latency when mammary tumours were produced by DMBA. It is currently known that TRF, α, γ and δ and tocotrienols, inhibited proliferation of estrogen receptor negative MDA-MB-435 human breast cancer cells with 50% inhibitory concentrations (IC50) of 180, 90, 30, and 90 mg/ml, respectively.

Prostate cancer

In a model where prostate cancer was induced by injecting PC-3 cells into nude BALB/c mice, it has been noted that the radiotherapy efficacy of prostate cancer can be increased with γ-tocotrienol and a prooxidant if the kidneys can be shielded (Kumar et al., 2006)

Liver cancer

Hepatoma cell proliferation is hindered by tocotrienol. Tocotrienol was absorbed by cancer cells more quickly than tocopherol, indicating that this may be a factor in why tocotrienol was so successful in eliminating hepatoma cells (Har and Keong, 2005; Sakai et al., 2004). γ-Tocotrienol inhibits the proliferation of human hepatoma Hep3B cells at lower concentrations and shorter treatment times than α-tocotrienol.

Gastrointestinal tract

Tocotrienol has been noted to inhibit telomerase activity of DLD-1 human colorectal adenocarcinoma cells in a time- and dose-dependent manner. The most potent inhibitory efficacy was shown by tocotrienol.

Cholesterol lowering

The α-tocotrienol form of natural Vitamin E may have considerable cholesterol-lowering effects rather than tocopherols. Mevalonate is produced by the ER enzyme 3-hydroxy-3-methylglutaryl CoA (HMG CoA) reductase and is then transformed into sterols and other compounds. Tocotrienols may reduce serum total and LDL cholesterol levels by preventing the hepatic enzyme HMG-CoA reductase from working through a posttranscriptional mechanism. But tocopherol has the reverse effect (increases this enzyme activity) (Qureshi et al., 2002).

SIGNIFICANCE OF ANNATTO TOCOTRIENOLS

Both the tocotrienol-rich lipid fraction and the geranilgeraniol found in annatto (*Bixa orellana* L) can be employed as nutritious additives in foods and beverages. In recent years, annatto has surpassed rice bran and palm oil and has become one of the richest sources of δ-tocotrienols. Additionally, annatto extract works well as an antioxidant agent and a preventative for lipid peroxidation by combining the effects of tocotrienols and bixin to shield unsaturated fats from oxidative damage. Tocotrienols have been utilised to lower the risk of cardiac disorders and have been claimed to have anticancer potential by postponing the growth of breast cancers. Due to its potential as an angiogenic inhibitor, the substance δ-tocotrienol is a prospective chemotherapeutic drug. It has been determined that annatto contains 140–147 mg of δ-tocotrienol per 100 g of dried seeds. This bioactive substance doesn't appear to be present in comparable concentrations in any other vegetable species. The amount of δ-tocotrienol and γ-tocotrienol extracted using supercritical CO_2 extraction was 146 4 mg/100 g of dry seeds and 20.1 mg/100 g of dry seeds, respectively.

DOWNSTREAM PROCESSING OF TOCOTRIENOLS

Tocotrienols occur in photosynthetic plants in varying amounts provide a useful source for these vitamin E forms. Researchers have created a wide range of techniques and methods for the extraction, analysis, identification, and quantification of tocotrienols from various sources, including solvent extraction, supercritical fluid extraction (SFE), column chromatography, thin layer chromatography, normal and reversed high performance liquid chromatography (HPLC), etc. A framework of the

numerous methods utilised to isolate and characterise tocotrienols is shown in Figure 11.

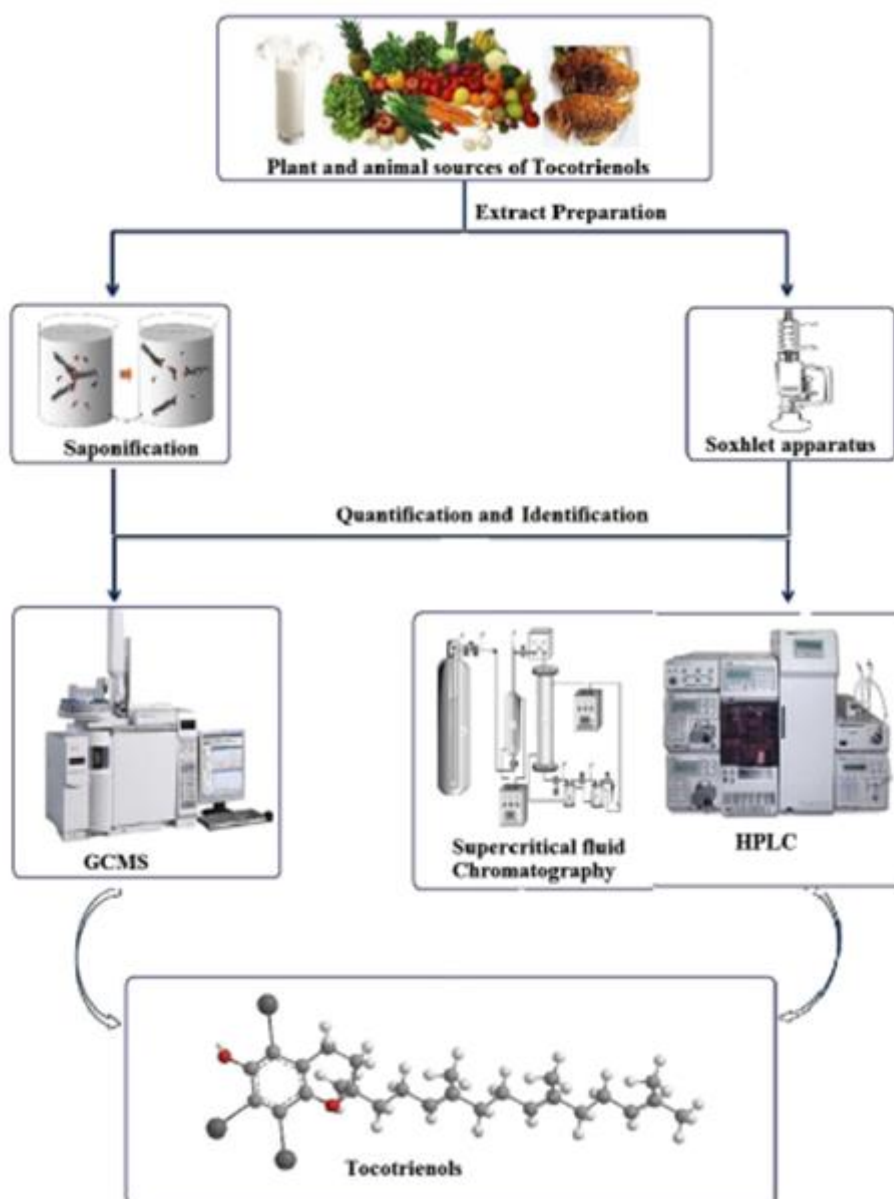

Fig. 11. **Downstream processing to obtain tocotrienol from hazelnuts**

(Haseeb et al., 2015)

EXTRACTION METHODS FOR TOCOTRIENOLS

Vitamin E has been extracted using a variety of techniques, including solvent extraction (direct, soxhlet, pressure-liquid, and supercritical fluid), enzymatic method,

chemical method (saponification, esterification), adsorption, molecular distillation, microwave-assisted extraction, membrane technology, and others. The fundamental goals of any purification operations are effectiveness, economy, and a sufficient threshold of purity and quantity. It works equally well for both large-scale production and sample preparation for characterisation. As a result, any procedure must have goals for purity, quantity, biological activity retention, and economy.

Soxhlet Extraction

Soxhlet extraction is a process in which the ground plant parts are packed in a Soxhlet apparatus and then extracted with the solvent of choice. From (Haseeb et al.,) tocotrienols from chopped hazelnuts were extracted using soxhlet extraction. From (US Patent 2011), the tocotrienols will be present in the lipid soluble fraction of the annatto seeds so that it can be extracted by using hexane.

Saponification

In the process of "saponification," triglycerides are converted into glycerol and a fatty acid salt known as "soap" by reacting with sodium or potassium hydroxide (lye). The triglycerides are often made of vegetable or animal fats. A hard soap is created when sodium hydroxide is used. A soft soap is produced when potassium hydroxide is used (Anne Marie, 2020). It has been regarded as one of the best method for the isolation of tocotrienols from plant as well as animal sources. In one experiment, the method involved an alkaline digestion prior to extraction of the unsaponifiable compounds with hexane. According to (Haseeb et.al), tocotrienols from chopped hazelnuts has been isolated by ethanol (2.5 mL), water (2.5 mL) and 10 M NaOH (0.5 mL).

SEPARATION, IDENTIFICATION AND CHARACTERIZATION OF TOCOTRIENOLS

Despite the fact that HPLC methods have been demonstrated to be suitable for the separation, determination, and isolation of αT oxidation products (Cuhna et al., 2006), only a few techniques allow the simultaneous separation of polar and nonpolar oxidation products, even when both the normal phase and the reversed phase are used for separation as the stationary phase. Due to the additional spectroscopic information that is provided by Diode array detection (DAD), Mass Spectrometry (MS),

Fluorescence (F), and electrochemical detection, these are the most common methods used to identify αT oxidation products. The simultaneous measurement of αT3 oxidation products and additional tocochromanol isomers, however, lacks a technique. The most popular technique for analysing tocochromanols is HPLC, which uses both normal (NP) and reversed phases (RP). The fundamental advantage of NP columns over RP columns for separation is their ability to entirely separate all isomers (Cert et al., 2000). These compounds are analysed using HPLC, which uses amperometric, UV, fluorescence, ELSD, and other types of detection. It is said that fluorescence detection is more sensitive and selective than UV. ELSD has been employed more frequently in numerous analytical laboratories as a result of its successful application in the analysis of numerous chemicals.

CHROMATOGRAPHY

Chromatography is a separation technique based on distribution between two phases: a stationary phase that is either solid or liquid and a mobile phase that is either liquid or gas. The stationary phase is percolated by the fluid mobile phase, which propels the sample. A large range of chemicals can be separated using several chromatographic techniques built on distinctive principles. A given chromatographic process may be run both in low and high pressure systems, although the basic principle remains same.

Thin Layer Chromatography (TLC)

One of the earliest and most popular chromatographic procedures for the identification, isolation, purification, and quantification of test samples is Thin Layer Chromatography (TLC). Utilizing TLC, tocotrienols of plant origin were successfully isolated. For the isolation, purification, and quantification of tocotrienols, several researchers have developed TLC methods (Abidi et al., 2009) (Chow et al., 2012). In one technique, a plant extract was spotted on silica gel, developed using hexane-ethyl acetate (92.5:7.5) or chloroform in one dimension and hexane-isopropyl ether (80:20) in another dimension, and then separated into six chemicals, including,, α, β, δ-tocopherols and, α, γ, δ-tocotrienols. Sample visualisation is often done under UV light (Chow et al).

Column chromatography (CC)

For the identification and separation of tocotrienols, researchers have used liquid-solid column chromatography (CC), as it more precisely separates unwanted contaminants than the TLC approach does (Kormann et al., 2011). To enable the quantitative recovery of tocols, silica gel, keiselgel, and hydrated florisil have all been widely employed as stationary matrix.

Gas Chromatography (GC)

Several researchers have created GC methods for the precise quantification of tocols in different oils (Hartman et al.,). A packed capillary column is used in GC equipment to provide high levels of detection, sensitivity, and component resolution. Additionally, a GC column is connected to a mass spectrometer or a Flame Ionisation Detection system (FID) to monitor common effluents or to identify and quantify structural components. Capillary columns have certain advantages over packed columns because they can more precisely, sharply, and sensitively detect molecules in the nanogram range. Additionally, it has thermal stability (Ballesteros et al.,). Before performing GC, the annatto sample(s) should either be acetylated or methylated to make it volatile.

Supercritical fluid chromatography (SFC)

SFC offers an advantage over gas chromatography and HPLC in the food sector since it uses an inert, low temperature supercritical carbon dioxide as the mobile phase eluent. In a single operation, SFC enables sample extraction, pre-concentration, chromatographic measurement, and preparative fractionation. SFC has been used to isolate a variety of tocopherols and tocotrienols (Choo et al.,). The experiment was performed on JASCO Model SUPER200 SFC system with a UV–Vis detector equipped with high-pressure flow cells. Columns used consisted of Lichrosorb silica 4.6×250 mm and Macherey-Nagel EC 250/4.6 Nucleosil 100-50 H diol column. SFC conditions included a temperature of 60 °C, pressure of 180 kg/cm2 and a flow rate at 3.0 mL/min for CO_2 and 0.12 mL/min for ethanol. The supercritical fluid in the study was supercritical CO_2. The column yielded a complete separation of tocol isomers following the sequence: αT, αT3, βT, βT3, γT, γT3 and δT3.

High Pressure Liquid Chromatography (HPLC)

For various analytical and preparative objectives, a mixture of substances can be separated using the chromatographic process known as HPLC. HPLC differs from conventional (or "low pressure") liquid chromatography in that it operates at much higher pressures (50–350 bar), whereas traditional (or "low pressure") liquid chromatography typically relies on gravity and runs at standard air pressure. Additionally, HPLC columns are constructed using extremely small sorbent particles (2–5 m in average particle size) that can withstand the high pressure created during the separation. A sampler, pumps, and a detector are commonly shown on an HPLC instrument's diagram. The sample combination is introduced by the sampler into the mobile phase stream, which then transports it to the column. The pumps push the mobile phase through the column at the correct flow and composition. It is possible to analyse the sample components quantitatively since the detector produces a signal proportional to the amount of sample component emerging from the column. In some HPLC equipment models, the mechanical pumps can combine various solvents in ratios that change over time, creating a composition gradient in the mobile phase. In comparison to normal pressure chromatography, HPLC has a number of distinct advantages, including high separation capacity, the ability to batch-analyze multiple components, superior quantitative capability and reproducibility, moderate analytical conditions, generally high sensitivity, easy preparative separation, and purification of samples. The examination of protocols in diverse sample matrices has made considerable use of both normal and reverse phase HPLC techniques. For the measurement of tocopherols and tocotrienols, various studies have used silica-based stationary phases, including Lichrosorb Si 60 (Thompson et al.,), Zorbax Si (Tan et al.,), Ultrasphere Si (Weber et al.,), etc.

Capillary Electrochromatography (CEC)

A hybrid technique called capillary electrochromatography (CEC) combines elements of capillary electrophoresis and HPLC (CE). Similar to CE, the separation is carried out in a capillary column with the mobile phase being propelled along by electro-osmotic flow, and the solutes being transported along the column carrying the stationary phase being either packed or bonded to the capillary wall. Many researchers have utilised it successfully to extract the tocotrienols from their numerous sources. The

method enables low solvent consumption high speed microanalysis. The instrument includes a CEC apparatus equipped with a diode array detector (290 nm) and a HP Chem Station software for system control. Three commercial CEC capillary columns (25 cm×100 µm I.D.) packed with 3 µm alkyl or aryl bonded silica and one 3 µm silica column obtained from a different source is widely used. These columns include CEC Hyposil C8, CEC-Hypersil C18, CEC-Hypersil phenyl and Unimicre CEC silica, 25 cm×75 µm I.D. The samples are run electro-kinetically onto the column at 10 kV for 10 s. Column temperature and separation voltage were maintained at 25 kV and 30 °C, respectively. CEC coupled with calibration data enables speedy sample analysis with adequate component resolution and enhanced detection sensitivity. CEC method can serve as a viable alternative to existing RP-HPLC method and can be used in the routine analysis of tocopherols and tocotrienols in oil samples (Abidi et al.,).

Mass spectroscopy (MS)

Mass Spectrometry Spectroscopic methods represent one of the main tools of modern chemistry. The majority of spectroscopic detection techniques are based on mass spectrometry, infrared, Raman, fluorescence, and NMR spectroscopy of 1H and 13C. Due to the challenges associated with ionising non-polar molecules, mass spectrometry has not been utilised frequently for the investigation of vitamin E.

Gas chromatography- Mass Spectroscopy (GCMS)

GC-MS is made up of two parts, Gas Chromatography and Mass Spectrometer. Gas Chromatography separates components from a sample mixture. Inside the mass chamber separated components are bombarded by a beam of high energy electrons and get ionized. High energy electrons cause the ionized molecule to lose an electron and forming a radical cations having net positive charge. This ions further breaks to fragment pieces because of its unstable nature. The fragments travel through the mass spectrometer, mass analyser and recorded by the detector according to their mass to charge ratio (m/z).

In order to detect or quantify tocol derivatives, GC-MS analysis is also used. Gas chromatography facilitates the identification of the analytes, although it necessitates time-consuming sample preparation steps, such as acetylation. When determining tocopherols and tocotrienols, GC was employed in matrices including vegetable oils, seeds, fruits, and other matrices of plant origin (Naz et al., Butinar et al., and Bhatia et

al., Frega et al.,). The sample was acetylated to make it volatile and to ensure that it would be easily detected by the GC-MS. By combining 2.0 mg of the sample with 600 μl of a 2:1 (v/v) pyridine-acetic-anhydride solution, the unsaponifiable material was acetylated. The solvent was then removed by nitrogen purging, the DCM washed, and the mixture was maintained at 4 °C for GC-MS analysis.

MATERIALS AND METHODS

The annatto seeds that is obtained from the plant *Bixa orellana* L were collected from farmland of Hyderabad. The seeds were slightly sorted to remove pieces of twigs, branches and leaves from the annatto seeds. The seeds were then kept away from exposure to light in opaque sacks. Then 300 g of the seeds were taken for Soxhlet extraction. To get course grounded powder the mixer grinding method was found to be effective, whereas manual grinding by mortar and pestle was not suitable for further processing.

Fig. 12. **Manually ground annatto seeds**

Fig. 13. **Mechanically ground annatto seeds**

CHEMICALS AND REAGENTS

All the organic solvents and chemicals used such as hexane, KOH, methanol, pyridine, aqueous alcohol, ethyl acetate, chloroform, sodium sulphate, phosphorous pentoxide, paraffin wax, silica gel were of analytical grade from Merck (Mumbai), Avra (Hyderabad).

PROCEDURE

Soxhlet extraction

Soxhlet extraction is a process in which the ground plant parts are packed in a Soxhlet apparatus and then extracted. The annatto seed sample of 300 g was taken and ground using a mixer grinder. It was then loaded in the Soxhlet apparatus and it was extracted with solvent, 5 times the volume of the sample, i.e. 1.5 litres of hexane.

Siphoning was done 20 times. The hexane soluble extract was then concentrated in the rotary evaporator and complete solvent was removed. The hexane insoluble extract which was sediment at the bottom was taken out and tried to dissolve them in methanol and ethanol. The solvent was then evaporated by heating on water bath and it was stored in polypropylene bags in dark atmosphere.

Fig. 14. **Soxhlet extraction of annatto seeds**

Fig. 15. **Concentrating hexane soluble extract in rotary evaporator**

Saponification

Saponification is a process by which triglycerides are reacted with sodium or potassium hydroxide for hydrolysis. It has been regarded as one of the best method for the isolation of tocotrienols from plant as well as animal sources. The method involved an alkaline digestion prior to extraction of the un-saponifiable compounds with hexane. The extract (5 g) was taken and to that, 1 g of KOH and 25 ml of methanol was added. It was then refluxed for 2 hours. After refluxing, the methanol extract was concentrated. The saponified matter was then kept in the desiccator for drying. The dried matter obtained was 5.2 g. The desiccants used were sodium hydroxide, phosphorous pentoxide and paraffin wax.

Fig. 16**. Saponification**

Soxhlet extraction

The dried saponified matter (5.2 g) was then extracted with hexane in the Soxhlet apparatus to separate the saponifiable and non-saponifiable matter. Since the tocotrienols was expected in the non-saponifiable matter, the hexane extract was collected and it was concentrated in the rotary evaporator. The remaining solvent was then evaporated by keeping on the water bath to get the saponified material with the yield of 3.98 g (76.5 %).

Fig 17. **Soxhlet extraction**

Thin layer chromatography

Thin layer chromatography (TLC) is one of the oldest and widely used chromatographic techniques for the identification, isolation, purification and quantification of assay samples and it will separate individual components by using finely divided adsorbent solid spread over a plate and liquid as a mobile phase.

The precoated TLC plates are used for experiments and developed spots were monitored and retardation factor (R_f) values are calculated by the formula,

$$R_f = \frac{\textit{Distance travelled by sample}}{\textit{Distance travelled by solvent}}$$

Procedure

With a pencil, a thin mark was made at the bottom of the plate to apply the sample spots. The sample was dissolved in methanol and chloroform. Both the samples were applied on the plate equidistantly. The TLC was done in different solvent systems as mobile phase, chloroform: methanol (9.5: 0.5); (1:1); (7:3). The solvent system was then changed to hexane: ethyl acetate in ratios (1:1), (9.5: 0.5), (9.25: 0.25), (9:1), (8:2) and (7:3). After the solvent reaches the solvent front, the plate is taken out of the solvent system, dried and kept in the iodine chamber to obtain visible spots.

Fig. 18. TLC of Saponified extract

Hexane: ethyl acetate (8:2)

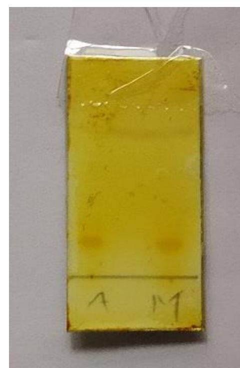

Fig. 19. TLC of Saponified extract

Hexane: ethyl acetate (9.5:0.5)

Column chromatography

Procedure

Silica impregnation of the sample

The silica was first kept inside the hot air oven for activation for one hour at 110 °C and then it was cooled to room temperature in a desiccator. The sample of 3 g was weighed and kept in a porcelain bowl and to it 5 ml of methanol was added. When the sample is completely dissolved in the solvent, 6 grams of activated silica (with mesh size 200-400) was added. It was then heated until the sample becomes powder form. The impregnated sample was kept closed away from moisture.

Fig. 20. **Column chromatography**

Elution

A column was taken and a layer of cotton was placed inside the column. Small amount of hexane was added to the column. A slurry of silica was prepared by dissolving 30 grams of silica in hexane. The slurry was poured into the column. It was allowed to settle. Once the silica was settled, the silica impregnated sample was transferred into the column. The solvent hexane was added sufficiently and it was left for few minutes. After complete settling, the elution was started. Each fraction of 50 ml was collected, concentrated in the rotary evaporator and monitored with TLC. Hexane was then added with 2% and 5% chloroform subsequently for elution. The fractions were concentrated and TLC was done. The spots were observed and the fractions with the spots having similar R_f value were pooled and concentrated on water bath.

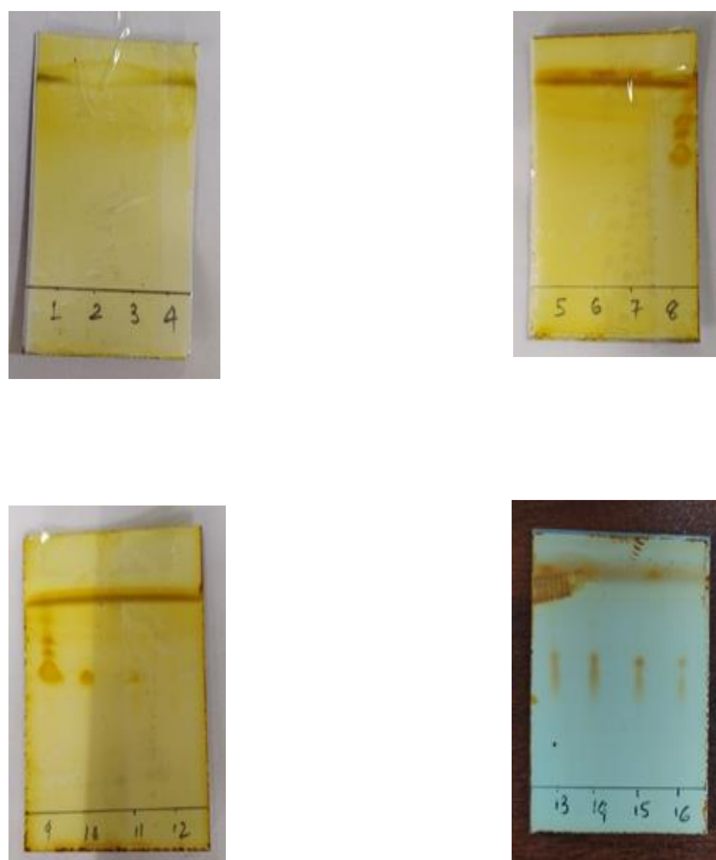

Fig. 21. **TLC plates of fractions 1-16**

The fractions 8 and 9 with 4 spots wer combined and made in to single fraction. Fractions 10 and 11 with single spots were combined seperately and after removal of solvent yield was found to be about 300 mg (10 %). All the other similar fractions were

combined and made into another fraction. The column elution was continued till all the components got eluted. The fractions 10 and 11 with single spots were taken for HPLC. This sample was also acetylated for GC-MS analysis.

High Performance Liquid Chromatography

HPLC separation was carried out on a Waters Spherisorb® 5µm Silica 4.0 x 250 mm analytical column using an isocratic mobile phase methanol: acetonitrile (55:45) with a flow rate of 1ml/minute. About 50 µg (50 µL) was taken and diluted to 400 µL with chloroform and from this preparation 10 µL was injected for HPLC. Peaks were detected at retention time 2.06 and 2.29 minutes (Fig. 25).

Fig. 22**. HPLC equipment**

The detection during HPLC analysis was carried out by PDA detector with the wavelength range of 200-400 nm under which the eluted components were detected and peaks are recorded.

Acetylation of samples for GC-MS

The sample of 50 mg was taken and it was dissolved in 100 mg pyridine and 50 mg acetic anhydride. The reaction was allowed to proceed at room temperature overnight (10-12 h). The excess reagents were removed by slight warming under a stream of nitrogen. The mixture was re-dissolved twice in dichloromethane and the solvent removed under stream of nitrogen. The acetylated products were transferred

into a sample vial (yield: 65 mg) and dissolved again in DCM, flushed with nitrogen and stored at 4 °C for GC-MS.

Fig. 23**. TLC of acetylated and non acetylated samples (Solvent system: Hexane: Ethylacetate 8:2)**

The R_f values under the optimized solvent system was found to be 0.2 cm for the starting material and 0.75 cm for acetylated sample. The acetylation efficiency was found to be about 80 % since 20 % found to be not acetylated as observed by TLC.

Column Chromatography

From the TLC, it was understood that few component got acetylated. Therefore to separate the acetylated component in pure form it was subjected to classical column chromatography using silica gel and for which the sample (0.065 g) was impregnated with silica (1 g) and eluted in a silica column with hexane to obtain the acetylated and non-acetylated fractions separately. Removal of solvent by evaporation on water bath gave about 30 mg of acetylated compound

Fig. 24. **TLC of fractions separated by column chromatography**

Gas Chromatography- Mass Spectroscopy

Analytical conditions for GCMS

The acetylated sample obtained by column chromatography (30 mg) was diluted with 200 µL of chloroform and injected 1 µL under the following condition.

The injection temperature was 220 °C and the interface temperature was 300 °C. The initial temperature was 60 °C held for 1 min and then ramped to 200 °C at the rate of 15 °C/min. It was then held for 1 min before the second ramp at the rate of 5 C/min to 300 °C. This was then held isothermally for 25 min.

The results obtained from the HPLC and GC-MS were reviewed with existing literature and the obtained compounds were identified.

RESULTS AND DISCUSSION

Annatto fruits contain the seeds which are known for the natural pigments namely the fat soluble bixin and water soluble norbixin. Besides this, the recent reports indicate that it contain significant amount of the vitamin E, in particular the tocotrienols among which the δ-tocotrienol is predominant. These compounds are present in minimum quantities from other sources, but still possess a wide range of biological activities. In the recent past, the cholesterol lowering and potential anticancer properties were reported for the δ-tocotrienol.

Under this context the present study was carried out to isolate the δ-tocotrienol from the annatto seeds on exploratory basis and the detailed results are presented below.

Preparation of extracts

The annatto seeds containing the coloured pigments, tocotrienols and other secondary metabolites are initially powdered using mixer grinder. The 300 g of powdered seed on extraction with Soxhlet gave about 3 % of the extract. This was saponified to facilitate the release of bound tocotrienols. Accordingly, 5 g of the sample was saponified for 2 h and saponified product was dried in desiccator.

Soxhlet extraction

The dried saponified product (5.2 g) was loaded in the thimble placed in a Soxhlet and extracted with hexane to get the δ-tocotrienol after 20 siphons the yield obtained was 76.5 %.

TLC analysis

The TLC analysis of the Soxhlet extract using solvent system in different proportion of hexane and ethyl acetate was attempted. Among which the 8:2 (hexane: ethyl acetate) was found to be more suitable giving better resolution showing one major spot with R_f value 0.5 besides other minor components.

Column chromatography

This was subjected to further purification by column chromatography method by taking 3 g of the Soxhlet purified extract impregnated with 6 g of activated silica

and separated by column chromatography by using hexane followed by 2 and 5 % chloroform, respectively. The 5 % chloroform gave single spot on TLC with 0.65 R_f value (Fig 21).

HPLC analysis for tocotrienols

The isocratic method with mobile phase methanol: acetonitrile in ratio 55:45 was used to determine the tocotrienol compounds in the sample. A Waters Spherisorb® 5 μ Silica 4.0 x 250 mm Analytical Column was used. Elite Lachrom 2000-HPLC system with Diode Array Detection (PDA/ DAD) was used.

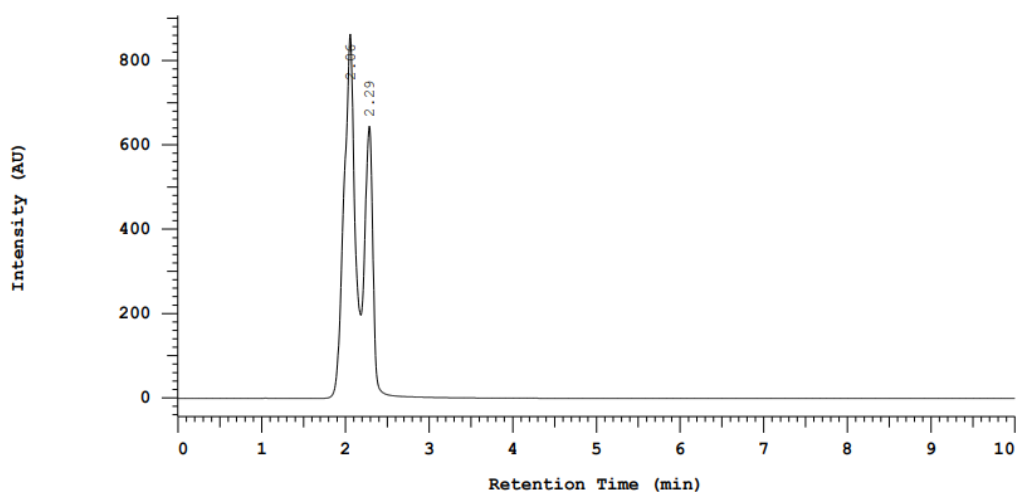

Fig. 25. **HPLC Chromatogram**

No.	RT	Area	Conc 1	BC
1	2.06	7774904	62.395	BV
2	2.29	4685881	37.605	VB
		12460785	100.000	

Two peaks were observed at retention time 2.06 minutes and 2.29 minutes simultaneously. These peaks were magnified and the absorbance wavelength in nanometres were found out.

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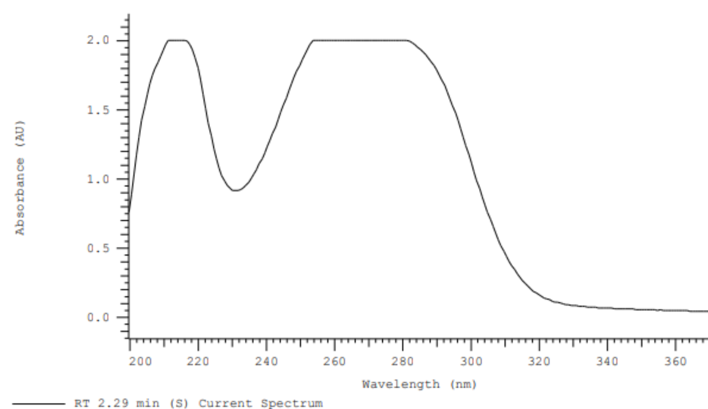


Fig. 26. **Spectrum at RT 2.29 min**

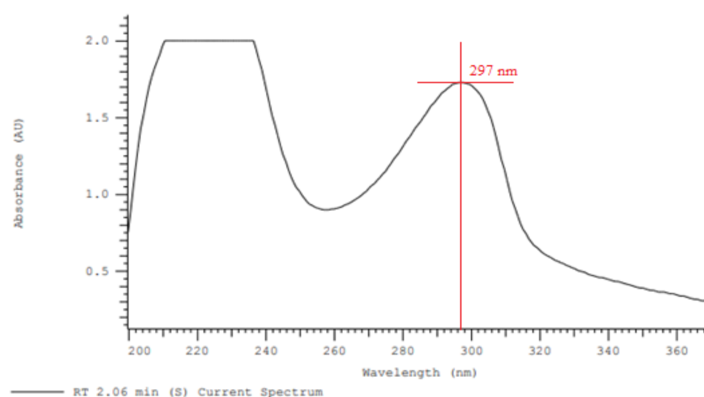


Fig. 27. **Spectrum at RT 2.06 min**

A clear and defined peak was observed at the retention time 2.06 min. This peak was extrapolited and the absorbance wavelength was found out to be 297 nm. This value was cross checked with the results of HPLC done in the literature (Lampi et al.,)

According to studies the absorbance wavelength of different tocols are listed below:

Type of tocol's	Absorbance wavelength (nm)
α-T	292 nm
β-T	296 nm
γ-T	298 nm
δ-T	298 nm
α-T3	292.5 nm
β-T3	294 nm
γ-T3	296 nm
δ-T3	**297 nm**

Comparing with the literature, the obtained compound was considered to be δ-tocotrienol. Since the absorbance wavelength of all the tocols are in near proximities, but were not observed in our PDA analytical detection.

Gas Chromatography- Mass Spectroscopy (GC-MS)

The acetylated samples were then send for GC-MS. **A**cetylation was done for the sample to make it volatile and for its efficient detection in the GC-MS.

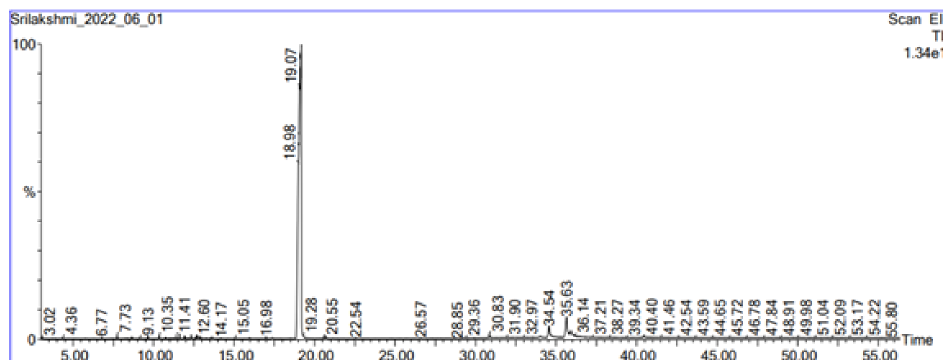
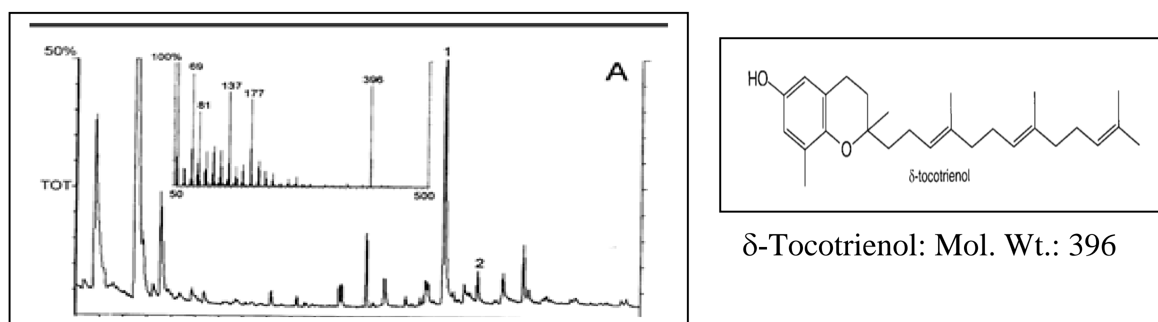


Fig. 28. **TIC of the acetylated product of annatto extract**



δ-Tocotrienol: Mol. Wt.: 396

Fig. 29. **Mass spectrum of δ-Tocotrienol** (reported, Massimo Mozzon, 1998)

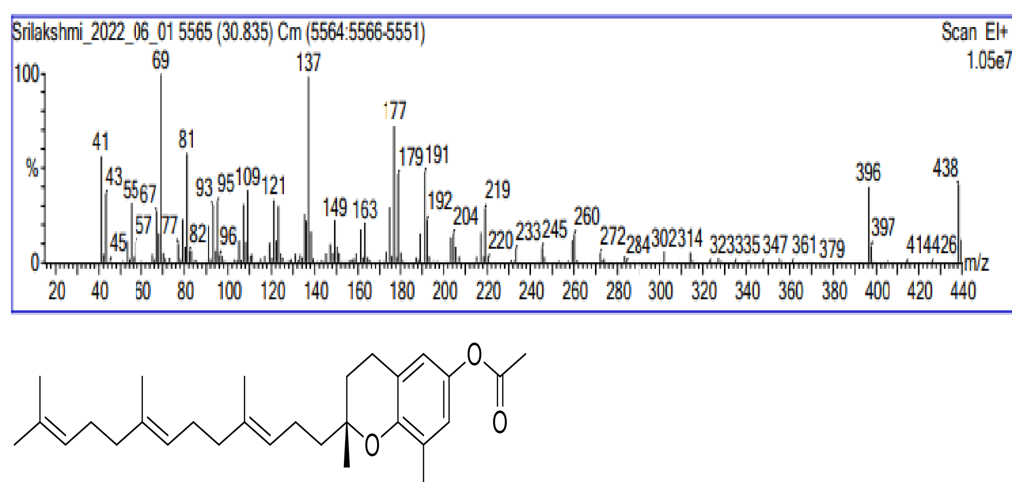


Fig.30. **Mass spectrum of δ-Tocotrienol acetate**; Mol. Wt.: 438 (acetylated product), as observed in our GC-MS analysis (RT 30.835).

Interestingly, the GC-MS TIC showed a major peak with RT 19.160 (83.667 %) which corresponds to the mass spectrum of geranyl geraniol with the molecular weight 290.5 m/z.

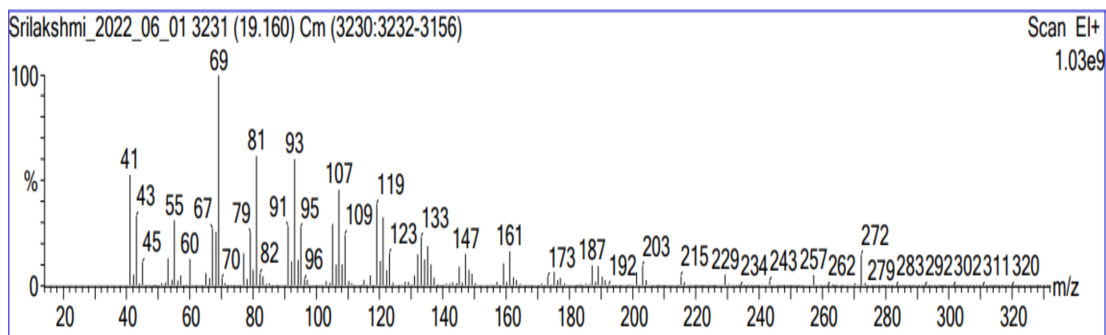


Fig. 31**. Mass spectra of acetylated compound**

This component was observed with 83.667 % in our analysis and this could be formed as a degraded δ-tocotrienol and rearranged to Geranylgerniol, probably during the acetylation process, which need to be studied with further experiments.

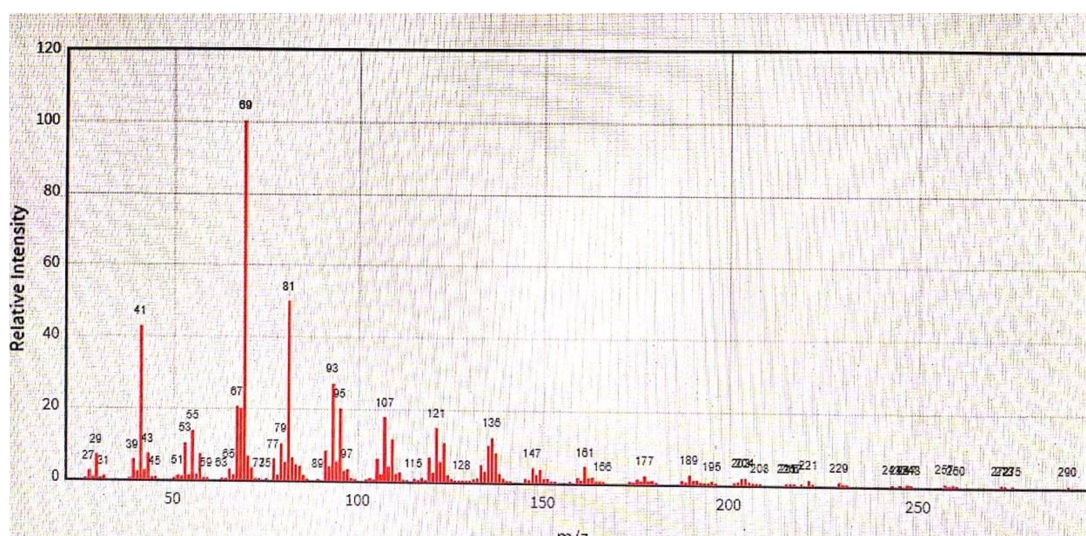


Fig. 32. **Mass of geranylgeraniol** (reported NIST Mass Spectrometry Data Center)

Table: GC-MS profiling of acetylated extract

Compound name	Retention time	Area percentage
Phenol, 2,4-bis-(1,1-dimethylethyl),	10.346	0.139
Aromadendrene, dehydro-	11.602	0.194
Cycloisolongifolene, 8,9-dehydro-9-formyl-	12.332	0.219
Neoisolongifolene, 8,9-dehydro-	12.752	0.133

Geranylgerniol	**19.160**	**83.667**
δ-Tocotrienol acetate	**30.835**	**0.336**
Ergost-5-en-3-ol, acetate, (3á,24R)-	33.986	0.346
Stigmasteryl acetate	34.541	2.027
Stigmastan-3,5-diene	35.627	3.885
methyl 3-oxolup-20(29)-en-28-oate	40.414	0.245

Thus, the preliminary work carried out on isolation of tocotrienols from annatto seeds by Soxhlet extraction and HPLC analysis showed the presence of δ-Tocotrienol which was evident from the PDA detection and absorbance maxima of 297 nm. Among the 2 major signal observed, 2.06 RT was corresponding to this and which showed relative percentage of 62.395 %, which is considered to be a significant proportion in the extract.

Therefore, further optimization of the extraction and isolation with supportive chromatographic and spectroscopic method will supplement the findings and subsequently provide the scope for developing process for the isolation of tocotrienols from the annatto seeds.

CONCLUSIONS

The present exploratory study on the isolation of tocotrienols from the annatto seeds resulted with extract by Soxhlet extraction using hexane followed by silica gel column chromatographic purification resulted with the tocotrienols rich conserve having two major components with RT 2.06 and 2.29 respectively as analysed by HPLC under standardized conditions.

- The HPLC analysis with PDA detection indicated the λmax value of 297 nm which corresponds to the reported value of δ-tocotrienol.
- This got eluted with the RT of 2.06 and showed the relative percent of 62.395 which is considerably a higher proportion.
- Further identification was carried out by doing a facile acetylation using pyridine and acetic anhydride followed by purification by column chromatography and subsequently by GC-MS analysis.
- GC-MS profile indicates 0.336% of δ-tocotrienol acetate wherein the major component was found to be geranylgeraniol (83.667%) with RT 19.160. This is attributed to the degraded product of δ-tocotrienol and rearranged to geranylgeraniol.

Thus, the preliminary work carried out on isolation of tocotrienols from annatto seed extract showed 62.395 % of δ-tocotrienol by HPLC, which is considered to be a significant proportion in the extract which is further validated by derivatization and GC-MS analysis.

Therefore, further optimization of the extraction and isolation with supportive chromatographic and spectroscopic method will supplement the findings and subsequently provide the scope for developing process for the isolation of tocotrienols.

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