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Bioactive Compounds: Historical Perspectives, Opportunities, and Challenges

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Mom's conventional wisdom of eating fruits and vegetables to lead a healthy life has evolved with scientific, fact-finding research during the past four decades due to advances in science of "Foods for Health". Epidemiological and prospective studies have demonstrated the vital role of fruits, vegetables, and nuts in reducing the risk of cancer and cardiovascular diseases. In recent years, several meta-analyses strongly suggested that by adding one serving of fruits and vegetables to daily diet, the risk of cardiovascular diseases will be decreased up to 7%. The multidisciplinary and partnership efforts of agriculture and medical scientists across the globe stimulated interest in establishing certain interdisciplinary centers and institutes focusing on "Foods for Health". While the consumption of various healthy foods continues, several questions about toxicity, bioavailability, and food−drug interactions of bioactive compounds are yet to be fully understood on the basis of scientific evidence. Recent research on elucidation of the molecular mechanisms to understand the "proof of the concept" will provide the perfect answer when consumers are ready for a "consumer-to-farm" rather than the current "farm-to-consumer" approach. The multidisciplinary research and educational efforts will address the role of healthy foods to improve eye, brain, and heart health while reducing the risk of cancer. Through this connection, this review is an attempt to provide insight and historical perspectives on some of the bioactive compounds from the day of discovery to their current status. The bioactive compounds discussed in this review are flavonoids, carotenoids, curcumin, ascorbic acid, and citrus limonoids.

KEYWORDS: Flavonoids; carotenoids; ascorbic acid; curcumin; limonoids; health benefits; clinical trials; milestones

INTRODUCTION

Diet and Health. Chronic diseases are the most prevalent cause of death in the world, led by cardiovascular disease followed by cancer, chronic lung diseases, and diabetes mellitus. A link between dietary cholesterol and atherosclerosis in animals was identified in 1908, and the relationship was found to be true in humans. Furthermore, epidemiological studies indicated that high serum cholesterol might have a strong correlation with increased risk of coronary heart disease (CHD). These findings led to the classical diet−heart hypothesis, which postulated the primary role of saturated fat and cholesterol in the development of atherosclerosis and CHD. In addition, initial studies suggested a direct relationship between high dietary fat intake and increased risk of breast and colon cancer. However, large prospective studies have not only addressed the effects of high dietary fat intake but also indicated the prevention of certain chronic conditions with a vegetable/fruit-rich diet. In recent years, the relationship between the consumption of specific foods and/or overall dietary patterns and the risk of CHD have been examined. Accumulating evidence from epidemiological, case control, and cohort studies suggests that a vegetable/fruit-rich diet may offer protection against chronic diseases. A study published in *JAMA* in 1999 by Joshipura et al. clearly demonstrated the need for higher consumption of fruits and vegetables to reduce the risk of certain diseases such as ischemic stroke (relative risk (RR) of 0.8 for extreme quantities). However, there was no apparent further reduction in risk beyond six servings per day. Another study has shown a reduced risk of myocardial infraction in women (RR of 0.62 for extreme quantities) consuming five servings of fruits and vegetables (*1*).

The above literature suggests that fruits and vegetables may have an important role in the maintenance of a healthy lifestyle. This observation also led to a million dollar question: "which components of fruits and vegetables may be responsible for the protective effects?" Furthermore, to understand these concepts, many clinical trials have been conducted using different bioactive compounds for the prevention of chronic diseases.

Human Clinical Studies on Selected Bioactive Compounds. Evidence from in vitro as well as in vivo experiments has

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Table 1. Selected Bioactive Compounds Tested for Clinical Trials

phytochemicals	health benefits	study design	refs
tea phenolics	antioxidant effects	study involved ~40 volunteers	*165*
quercetin	proliferation inhibition ability through, tyrosine kinase	study resulted in inhibition of tumor through tyrosine kinase inhibition (14 subjects)	*166*
β-carotene and lycopene	prostate cancer prevention	phase II randomized study (26 newly diagnosed prostate cancer subjects)	*167*
rutin	antioxidant	randomized single-blind placebo-controlled trial (18 female subjects)	*168*
isoflavones	prostate cancer prevention	phase II randomized, placebo-controlled clinical trial (242 prostate cancer subjects)	*169*
ascorbic acid	prevention of advanced malignance	intravenous administration of ascorbic acid (24 subjects)	*148*
epigallocatechin gallate (EGCG)	anticancer	protection against mutagenicity and genotoxicity, inhibition of biochemical markers of tumor initiation, inhibition of biochemical markers of tumor promotion, effects on detoxification enzymes, trapping of activated metabolites of carcinogens, and antioxidant and free radical scavenging activity	*170*
curcumin	anti-inflammatory and anticancer	Cox-2 and NFκB inhibition	*171*
ellagic acid (pomegranate juice)	prostate cancer	delay in proliferation of prostate specific antigen (PSA)	*172*
fructose and oroanthocyanidin (cranberry)	urinary tract infections	acidify urine and prevent adhesion of microbes in urinary tract	*173*
quercetin and apigenin	cardiovascular risk	antiaggregators effect	*174*
omega-3 and -6 fatty acids, green tea, licorice, quercetin, shark cartilage, curcumin	anticancer	antiangiogenesis	*175*, *176*
podophyllotoxin (mayapple)	breast cancer	induction of apoptosis	*177*

convincing results regarding potential health benefits of plant-derived compounds commonly known as bioactive compounds. These are present in different diets and have been speculated and proven to be the causal factors for the various health-maintaining properties. Bioactive compounds are secondary metabolites present ubiquitously in the plant kingdom and are considered as nonnutritional but vital ingredients for the maintenance of human health. Flavonoids, curcumin, carotenoids, and ascorbic acid are thoroughly investigated for their human health-maintaining properties at different stages and reached human clinical studies, which confirm the potency of these bioactive compounds. In recent years, several bioactive compounds have been subjected to investigation on their potential antioxidant, anticancer, and anti-inflammatory activities using different in vitro and in vivo models as well as clinical trials (**Table 1**). Among millions of characterized bioactive compounds, very few compounds have been tested on the basis of their ancient knowledge to current clinical applications (plant to patients). This review will address the current status of a few bioactive compounds such as phenolics (curcumin and flavonoids), carotenoids (β-carotene), vitamin C, and citrus limonoids.

FLAVONOIDS

Flavonoids were first reported by Robert Boyel's *Experiments and Considerations Touching Colors* in 1664 (*2*). Later during the 1800s the red color of wine and the blue pigment of corn flower were ascribed to the presence of flavonoids and anthocyanin pigments (*3*, *4*). Structural classification and the occurrence of the flavonoids in fruits and vegetables are shown in **Figure 1**.

The major research events of flavonoids are presented in **Table 2**. Medical interest in flavonoids stems back to the early 1900s when Russian scientists Rusznyak and Szent-Gyorgyi identified that fractions of Hungarian red pepper and lemon juice rich in flavonoids have the ability to improve capillary resistance. They further proposed that flavonoids have vitamin-like properties and termed flavonoids "vitamin P" (*2*, *5*). Later hesperidin and eriocitrin were identified as the active principles in red pepper and lime juice fractions. Initial reports demonstrated that flavonoids can decrease capillary fragility (*6*, *7*). The effect on capillary fragility was evident because hesperidin was the predominant component in the experiment. In addition, similar activity was reported from rutin during 1958 (*8*). The name "vitamin P" was discontinued during the 1950s (*9*); however, these observations led to an intense interest in elucidating and identifying more flavonoids in nature.

Chemistry and Analytical Techniques. The idea of flavonoids as bioactive components necessitated their purification from various plant species. To achieve this goal, chromatographic techniques were developed, which were employed to obtain relatively pure flavonoids. Adsorption chromatography emerged as one of the prominent methods for the separation and purification of flavonoids in 1950s. Several researchers have reported successful separation of closely related flavonoids by adsorption chromatography (*10*). Numerous flavonoids were successfully separated on silica columns by elution with combinations of different solvents such as chloroform, acetone, and methanol (*11*). Consequently, several new flavonoids such as querecetin, isoquercetin, and kaempferol were identified (*12*). Meanwhile, flavonoid profiles were generated for various plants including strawberries, citrus, and grapes (*12*−*14*).

Identification of new flavonoids led to the development of new analytical techniques for individual flavonoids. Several techniques were developed for the identification and quantification of flavonoids, and many of these techniques were based on fluorescence and ultraviolet detection. Moreover, the identification of flavonoids by paper chromatography upon reaction with Benedict's reagent was discovered (*12*). Identification of flavonoids by these methods further led to the development of thin layer chromatography and paper chromatographic techniques for separation and identification (*12*, *13*).

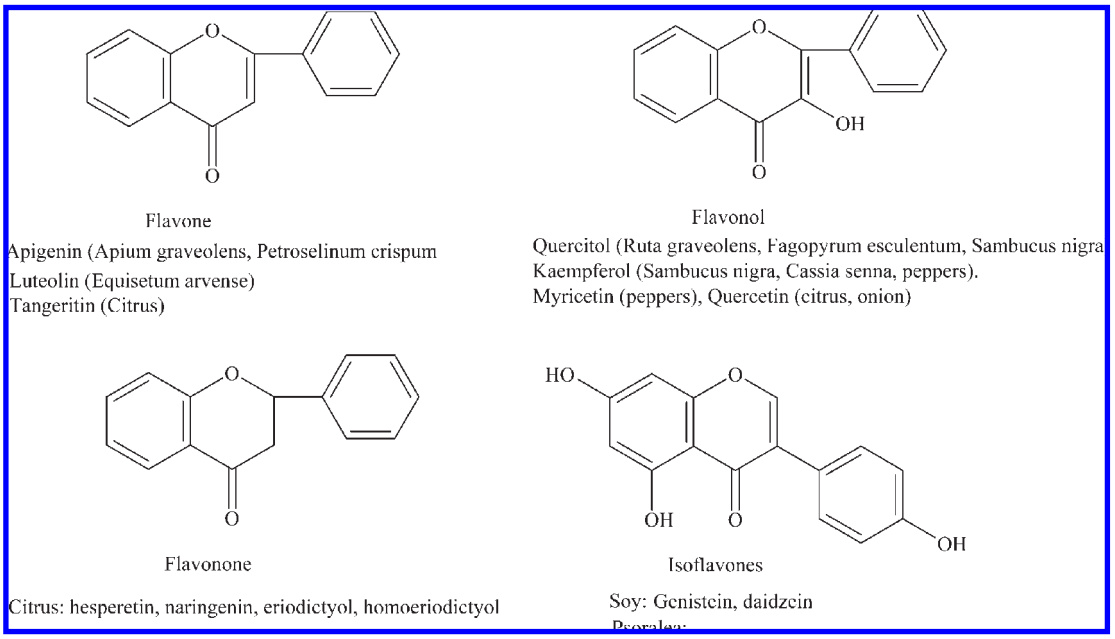

Figure 1. General structures of flavonoids and their occurrence in fruits and vegetables.

Table 2. Chronological Progress in Flavonoids Research

year	development	refs
1936	flavonoid-rich fractions shown to improve capillary fragility	*2*
1936	flavonoids are named vitamin P	*5*
1949	antioxidant activity of rutin	*178*
1950	the name vitamin P discontinued	*9*
1951	reaction with Benedict's reagent applied for flavonoids detection and analysis	*12*
1952	thin layer chromatography and paper chromatographic methods	*13*
1952	adsorption chromatography technique	*10*
1952	purification of quercetin and isoquercetin	*179*
1952	identification of kaempferol	*180*
1952	anti-inflammatory property	*181* and refs cited therein
1956	esterogenic activity	*182*
1958	structure of flavonoid established	*21*
1960	separation of flavonoids on silica column	*11*
1965	application of mass spectrometry for structure elucidation	*183*
1976	first use of HPLC for flavonoids analysis	*20*
1985	studies on flavonoids benefits in cardiac health—the Zutphen Elderly Study	*30*
1988	flavonoids are scavengers of superoxide	*184*
1997	flavonoids behave as both antioxidant and pro-oxidant	*185*
2004	pharmacokinetics of flavonoid reported	*186*
2005	anticancer activity of flavonoids involves inhibition of fatty acid synthesis	*187*
2008	meta-analysis of randomized clinical study reported on flavonoids	*188*

High-performance liquid chromatography (HPLC) techniques were initiated and subsequently refined in the 1970s (*15*). The first application of HPLC in flavonoid analysis was published in the *Journal of Agricultural and Food Chemistry* in 1976 (*15*). Since the inception of the chromatographic techniques, flavonoid detection has been reported and reviewed by several authors (*16*, *17*). During the early phase of development, normal-phase chromatography was the most commonly used technique for the separation of flavonoids (*17*). Later, polymethoxylated flavones were separated from orange and tangerine peels using a similar technique (*18*).

Studies on both biological activity and analytical science were conducted extensively for flavonoids (*19*, *20*). Moreover the biogenesis of the flavonoids was elucidated using ^{14}C tracer techniques (*21*). The benzene ring to which the pyran ring (anthocyanidin) or the pyrone ring (flavone) is attached has been designated the "A-ring" and the other benzene ring the "B-ring" (*19*, *20*). Additional structural complexity is introduced by the common occurrence of flavonoids as the *O*-glycosides in which one or more of the flavonoid hydroxyl groups are bound to a single or multiple sugar units by an acid-labile hemiacetal bond (*18*).

Biological Activities. Estrogenic and antiestrogenic properties of flavonoids were extensively investigated during the decades of the 1970s and 1980s (*22*). Flavonoids such as genistein, daidzein, apigenin, kaempferol, and naringenin were found to possess antiestrogenic activity by inducing estrogen-receptor-mediated signaling (*22*). The major discovery of the "French paradox", which addresses the potential health benefits of polyphenols in wine and related alcoholic beverages, incited researchers to focus on flavonoids (*23*). As of today, more than 5000 flavonoids have been identified and characterized and some have become clinically useful. Flavonoids have been proven to have an effect on antioxidant activity and the prevention of cardiovascular disease and free radical mediated diseases (*24*). Among the human clinical studies conducted using flavonoids, there have been numerous accounts demonstrating the positive effect that flavonoids have on antioxidants (*25*), coronary heart diseases (*24*), hemorrhoidals, rosacea (*26*), and Alzheimer's disease (*27*, *28*).

Over time, many scientists became interested in the use of flavonoids as antioxidants. Several researchers examined the antioxidant properties of flavonoids during the early 1990s (*29*, *30*). Consequently, various flavonoids including quercetin, morin, and baicalein were identified as strong antioxidants (*31*). It was suggested that the unsaturated ketonic structure of the pyrone ring, a free hydroxyl group in position 3, and free *o*-dihydroxy (3′, 4′) grouping in the side phenyl nucleus contribute to the antioxidant activity of quercetin (*32*).

Several epidemiological studies have shown that the consumption of flavonoids and diets rich in flavonoids are beneficial in the prevention of heart diseases (*29*). In recent years, a tremendous

Table 3. Major Clinical Studies Conducted Using Flavonoids for Health Benefits

year published	trial conducted	major outcome of the trial	ref
1996	phase I trial on quercetin for in vivo tyrosine kinase inhibition activity	quercetin was safely administered, and inhibition of tyrosine kinase was observed along with antitumor effect	*166*
1996	flavonoid consumption and mortality in Finland Cohort Study	people with low intake of flavonoids are at high risk of coronary disease	*189*
1996	intake of flavonoids and risk of coronary heart disease (CHD) in male health professionals	data do not strongly support the relationship between CHD and flavonoid intake; however, there were some positive responses	*29*
1999	intake of flavonoids and risk of CHD in postmenopausal women	broccoli has shown strong benefit in preventing CHD	*190*
2000	micronized flavonoids for control of bleeding from acute internal hemorrhoids	micronized flavonoids had rapid cessation of bleeding and a reduced risk of relapse	*191*
2001	dietary catechin in relation to CHD death among postmenopausal women	apple and wine catechins are found to be more beneficial, and other catechins will also help in prevention of CHD	*192*
2002	consumption of dietary antioxidants in association with risk of Alzheimer's disease (flavonoids were one of the treatments)	vitamins C and E showed significant benefits	*27*
2003	antioxidant effect of tea bioactive compounds (flavonoid is one of the major constituents)	bioactive compounds protects DNA against oxidative damage	*165*
2003	micronized flavonoids in pain control after hemorrhoidectomy	micronized flavonoids reduced the pain and severity of reaction and intramuscular analgesic requirement	*193*
2003	flavonoid consumption and CHD mortality	intake of high amount of flavonoid along with tea and red wine are associated with reduced risk of CHD	*194*
2005	flavonoids of *Chrysanthellum indicum* for the treatment of rosacea	flavonoids of the plant is well tolerated and help in prevention of moderate rosacea	*26*
2005	combining micrometerized flavonoids with infrared photocoagulation for treatment of hemorrhoidal disease	five days of treatment combining micronized flavonoids with infrared photocoagulation significantly reduced bleeding status	*195*
2008	flavonoids, flavonoid-rich foods for prevention of cardiovascular risk	flavonoids of soy and cocoa are helpful in prevention of cardiovascular risk	*188*

effort has been placed toward elucidating the biological activities of flavonoids in clinical trials. **Table 3** summarizes some of the major clinical studies conducted using flavonoids and the outcomes of those studies. In a recent cohort study, seven high-quality epidemiological studies linking flavonoids to cardiovascular disease (CVD) were evaluated. The Rotterdam Study (*33*) and the Zutphen Elderly Study (*30*) observed an inverse correlation between the intake of these flavonoids and CVD incidence. After a 5.6 year follow-up, the Rotterdam Study (*33*) found a 65% reduction in the relative risk for nonfatal myocardial infarction (MI) in a cohort of 4807 subjects 55 years old, but only a nonsignificant 7% reduction in the risk for fatal MI. The Zutphen Elderly Study, a 10-year follow-up of 805 men 65−84 years old (*33*), reported a significant reduction in the relative risk of CVD mortality in the highest quartile of daily intake (30 mg) of flavonols and flavones; this level of intake also predicted a 38% lower incidence of first MI. This association was not statistically significant. It is important to note that these data were limited only to the intake of quercetin. Furthermore, CVD mortality was significantly reduced by 21% in those who had a daily intake of at least 4 mg of quercetin. These studies demonstrated a positive effect of flavonoids consumption and strengthened the claim that higher intake of fruits and vegetables is beneficial for health.

Flavonoids are well-known for their antioxidant activity due to their structural features, such as multiple hydroxyl groups and number of double bonds. There is evidence showing flavonoids can interact with intracellular signaling molecules such as protein kinase and lipid kinase signaling cascades, which results in alteration of the phosphorylation state of target molecules resulting in modulation of gene expressions (*34*). Inhibition of protein kinase is known to be beneficial in cancer, inflammation, neurodegeneration, and other proliferation-related symptoms. Flavonoids are also known to bind to the ATP binding sites of a number of proteins including mitochondrial ATPase, calcium plasma membrane ATPase, protein kinase A, and protein kinase C (*34*). Apart from these, the interaction with benzodiazapine binding sites of GABA-A receptor is also reported (*35*). Despite all of these potentials, flavonoids are known for their lesser bioavailability from dietary supplements. Only aglycones can cross the cell wall of the gut; because most of the flavonoids occur as β-glycosides, they are not absorbed unless the β-glucosides link is broken. Hydrolysis can occur to break this bond only with the help of gut microorganisms; hence, the bioavailability of most of the flavonoids from the food matrix is limited (*36*). There are a number of research papers explaining the mechanisms of individual flavonoid compounds for inhibition of cancer; however, bioavailability from the food matrix has not been addressed extensively. Research in this direction will be of great potential to enhance the utility of flavonoids for effective prevention of chronic diseases.

Carotenoids. Carotenoids belong to a class of fat-soluble natural compounds found in plants, algae, and photosynthetic bacteria. The history of carotenoids dates back to 1831 when Wackenroder first isolated a pigment from carrots and named it "carotin" (*37*). Then, carotenes were widely discovered in plants (*38*). Six years later, in 1837, Berzelius named the yellow pigments from autumn leaves xanthophyll. In 1910 Tswett named the entire class of related pigments carotenoids (*39*). Strain (1938) used the name carotene for the hydrocarbon, and xanthophyll for oxygenated derivatives of hydrocarbons (**Figure 2**) (*40*−*42*).

Chemistry and Analytical Techniques. The Russian botanist, Tswett (1906, 1911), is credited for the separation and purification of the carotenes and xanthopylls for the first time. Tswett invented chromatography for the separation of the leaf pigments, that is, green chlorophylls, and yellow-to-orange carotenes and xanthophylls (*43*). During the first half of the 20th century, research primarily focused on chemical properties of some carotenoids found in animal tissues (*44*). It was shown that carotenoids contain basic isoprene units (*44*). However, it was also realized that carotenoids differ from terpenes. Whereas terpenes are products of simple polymerization or condensation of isoprene units, genesis of carotenoids is usually achieved by dehydrogenation to form double bonds. Higher order carotenoids derived from tetraterpenes usually form small molecules such as farnesol or phytol, which then combine to form tetraterpenes (*45*).

The sequence of the biochemical reactions that constitute the pathway of carotenoid biosynthesis in plants was reasonably well

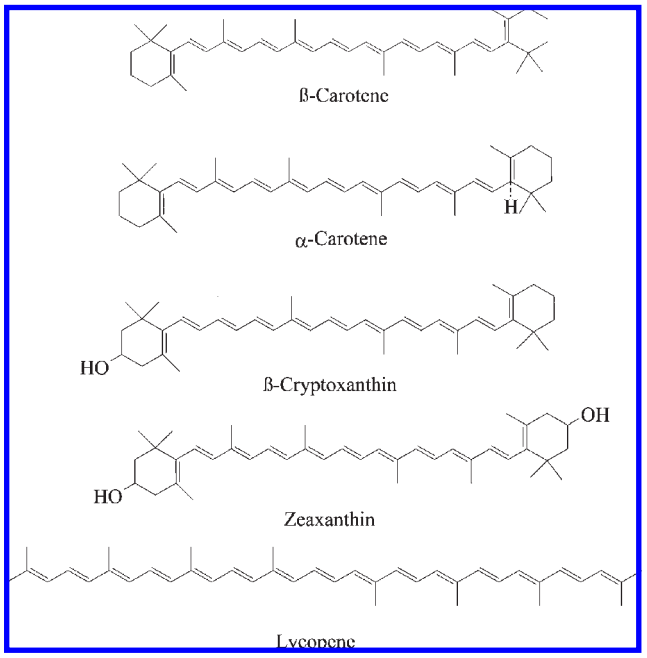

Figure 2. Structures of carotenoids and xanthophylls.

established by the early to mid-1960s from perceptive analysis of the intermediates that accumulated in naturally occurring mutants (*40*−*42*). In subsequent years, the characterization of many of the reactions in cell-free systems and in vivo studies using radiolabeled precursors provided further support and addressed many details. In later years, genes and cDNAs encoding nearly all of the enzymes required for carotenoid biosynthesis in green plants were identified and sequenced, and their products were characterized. Recent research has focused mainly on higher expression in plants and the genetic aspect of these carotenoids for their best possible utilization in nutraceuticals and pharmaceuticals. In 1950, a newly developed method to synthesize β-carotene facilitated further research (*46*).

The second half of the 20th century was marked by increased interest in bioactive compounds research. A search on carotenoids in PubMed showed about 18000 research papers on carotenoids as compared to a total of about 1200 papers published between 1900 and 1950. The major focus during this period was identification of different carotenoids in different plant species as well as their biological activities. Several analytical techniques were developed between 1950 and 1990 for the study of carotenoids (*47*). After the 1990s, there was a rapid development in analytical techniques for carotenoid analysis; the most famous of these methods were HPLC and mass spectroscopic techniques. In a study published in 1996, the Swartz group demonstrated that a C30 column was more selective for carotenoid analysis than a C18 column (*48*).

Biological Activities. After identification of vitamin A as an essential requirement for livestock, Steenbock in 1919 reported a close correlation between yellow pigments and vitamin A (*44*). Moreover, carotene and xanthophylls were found in the retina (*49*) and blood plasma (*44*). Biological activities of β-carotene using cell culture models are summarized in **Table 4**. With the discovery of carotenoids in animal tissues, increased attention has been directed toward the quantitative study of plant and animal pigments in relation to their vitamin A activity. Moore in 1930 reported that β-carotene is converted to vitamin A and may show provitamin A activity (*43*). Euler reported that provitamin A like activity of yellow corn is due to carotenes, $C_{40}H_{56}$ (*47*). Later, Kuhn and Grundman isolated cryptoxanthin, $C_{40}H_{56}O$, which also has provitamin A activity (*45*). Zeaxanthin, $C_{40}H_{56}O_2$, a xanthophyll isolated chromatographically by Karrer (*50*), was found to be completely devoid of growth-promoting activity when fed to rats (*49*). Lutein, the xanthophyll found in alfalfa meal, was not found to be a precursor of vitamin A (*44*).

Table 4. Effect of β-Carotene on Various Cells and Cellular Systems

cells	activity	reference
mammary and mouse	inhibition of alveolar lesions	*196*
Chinese hamster ovary	reduction of sister chromatid exchange	*197*
human lymphocytes	inhibits proliferations	*198*
human peripheral blood mononuclear	prevent decrease in antigen expression	*199*
human polymorphonuclear leukocytes	inducing secretion of cytotoxic cytokine	*200*
melanoma	increases differentiation reduces adenylate cyclase	*28*

Numerous studies were initiated to investigate the potential effect of carotenoids on human health during and after the 1990s (**Table 5**). Certain carotenoids, such as lycopene, have proven to be extremely beneficial in pancreatic and prostate cancers. In a study conducted by Nkondiock et al. in 2005, lycopene was found to be positively associated with the control of pancreatic cancer (*51*). However, in the same study, scientists were unable to find a positive effect of other carotenoids such as α- and β-carotene, β-cryptoxanthin, and lutein plus zeaxanthin. The concentrations of carotenoids such as β-carotene, lutein, zeaxanthin, and β-cryptoxanthin were found to be inversely related to the incidence of endometrial cancer in a multicentric case control study (*52*). The study was conducted between 1992 and 2006 with 454 women with endometrial cancer and 908 women as control. The odds ratio (OR) for these carotenoids was below unity, indicating an inverse relationship between specific carotenoid intake and disease incidence. Furthemore, carotenoids seem to play a role in diabetes control. Montonen et al. (*53*) showed that β-cryptoxanthin intake was significantly associated with a reduced risk of type 2 diabetes (RR 0.58).

Most of the early studies have shown that β-carotene may be helpful in the prevention of various types of cancer on the basis of in vitro and in vivo results (*54*). β-Carotene has shown significant radical scavenging activity in both in vitro and in vivo systems. This activity was thought to be useful in the inhibition of proliferation of cancer cells, anti-inflammatory, and other beneficial effects. Some of the major milestones in the carotenoids are summarized in **Table 5**. On the basis of the antioxidant activity and provitamin A potential, β-carotene was subjected to both animal studies and human clinical trials to understand its benefits on cancer prevention. Only a few studies have shown positive results indicating benefits in different cancer prevention. Studies conducted during the 1990s and later have shown no beneficial effects, and one of the studies involving smokers has shown an adverse reaction upon consumption of β-carotene.

Lycopene, a carotenoid present in many fruits and vegetables including tomato, grapefruit, and watermelon, is a symmetrical tetraterpene assembled from eight isoprene units. Lycopene was first investigated by Millardet in 1876, and a later investigator claimed that it was identical to carotene (*55*). However, in 1903, Schunck demonstrated that lycopene is distinct from carotene and named it "lycopene" (because it was a red pigment of the tomato *Lycopersicon*) (*55*). Isolation procedures for lycopene were first reported in 1910 (*56*), and the structure of the molecule was identified (*57*). Di Mascio and his co-workers studied the singlet oxygen quenching ability of different carotenoids,

Table 5. Major Milestones in the Area of Carotenoids Research

year	development	refs
1831	Weckenroder coins the term "carotene"	*201* and refs cited therein
1887	Arnaud describes the presence of carotenes in plants	*202* and refs cited therein
1906−1911	invention of chromatography for purification of xanthophylls by Tswett	*39* and refs cited therein
1914	Palmer and Eckles discover the existence of carotene and xanthophylls in human blood plasma	*203*
1931	Moore demonstrates that β-carotene will convert itself into colorless vitamin A in the liver	*204*
1934	Kuhn and Gurdman show corn is rich in cryptoxanthin	*47*and refs cited therein
1934	zeaxanthin isolated chromatographically	*46*
1934	vitamin A and carotenoids identified in retina	*45*
1935	discovery of vitamin A in animal tissues	*205*
1938	Strain names carotenes and xanthophylls	*206*
1938	Euler reports vitamin A like activity of yellow corn due to carotenes	*47* and refs cited therein
1941	discovery of carotenoids in human metabolites	*45*
1950	Isler and colleagues develop a method for synthesizing β-carotene	*207*
1959	quantitative paper chromatography of carotenoids	*208*
1965	mass spectrometry for structural determination by Schweiter	*209* and refs cited therein
1966	Expert Committee on Food Additives from FAO and WHO accepted β-carotene for use in food	*210*
1976	first use of NMR	*211*
1978	first use of HPLC for carotene determination	*212*
1981	Peto et al. exploit the possible benefits of carotenoids in cancer	*213*
2002	β-carotene can help in prevention of ARMD	*214*
2005	lycopene inversely associated with pancreatic cancer	*51*

tocopherol and bile acids (*54*). Their investigation revealed that lycopene is the most effective singlet oxygen quencher, firmly establishing the antioxidant potential of lycopene. In addition to its antioxidant properties, lycopene demonstrated an array of biological effects including cardioprotective, anti-inflammatory, antimutagenic, and anticarcinogenic activities (*58*). In recent years, lycopene has emerged as a potential chemopreventive agent. Several epidemiological studies have shown an inverse relationship between lycopene intake and cancer incidence. The data were derived from the Health Professional Study that reported a significant inverse relationship between lycopene consumption and prostate cancer based on data from 1986 to 1992 (*59*). Later, the study elaborated the analysis on a bigger time frame of 1986−1998 and again found a significant effect of lycopene intake on prostate health (*60*). In another epidemiologic study conducted by Erhardt et al. in 2003 (*61*), the authors found that patients with a high incidence of colorectal cancer have a low median level of plasma lycopene and β-carotene. These studies seems to suggest a potential role of carotenoids in colon and prostate cancer prevention.

Furthermore, several in vivo and in vitro studies have demonstrated a molecular mechanism explaining chemopreventive ability of lycopene. Lycopene is known to induce apoptosis and cell cycle arrest and to inhibit cell invasion, metastasis, and PDGF-BB-mediated signaling (*62*). Possible mechanisms of action to prevent cancer by lycopene include one or more of the following: (a) inhibition of growth and induction of differentiation in cancer cells by modulating the expression of cell cycle regulatory proteins; (b) modulation of the IGF-1/IGFBP-3 system (*62*); (c) up-regulation of tumor suppressor protein Cx43 and increased gap junction intercellular communication (*63*); (d) modulation of redox signaling; (e) prevention of oxidative DNA damage (*58*); and (f) modulation of carcinogen metabolizing enzymes(*64*).

Carotenoids were considered to be one of the best antioxidants on the basis of in vitro evidence before the 1990s. Research explaining the pro-oxidant effect of β-carotene and other carotenoids under specific oxygen concentration and under oxidative stress condition explained the possible cause for negative results in clinical trials involving the smoker's group. β-Carotene and other carotenoids are known to act as antioxidants in the presence of co-antioxidant and once the co-antioxidant is exhausted, these compounds act as pro-oxidants (*65*). Because most of the dietary matrix contains more than one antioxidant, the pro-oxidant behavior of carotenoids cannot be ignored. Whereas the outcomes of clinical studies on the benefits of carotenoids in cancer prevention are ambiguous, studies related to either diets rich in carotenoids or those combining β-carotene with vitamin E and ascorbic acid seem to show positive effects in the prevention of cancer. On the basis of the outcome of numerous clinical researches, it is clear that cancer prevention benefits associated with vitamin A are warranted, but not the direct benefits of β-carotene.

CURCUMIN

Curcumin, the yellow pigment of turmeric (*Curcuma longa*), is produced industrially from turmeric oleoresin and chemically known as 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione. Curcumin has been known for potential health benefits from ancient times (*66*) and is currently used as a key ingredient in both cosmetics and pharmaceuticals.

Chemistry of Curcuminoids. Curcuminoids refer to a group of phenolic compounds present in turmeric. Three curcuminoids, curcumin, demethoxycurcumin, and bisdemethoxycurcumin (**Figure 3**), were isolated from turmeric. These putative bioactive compounds impart the characteristic yellow pigmentation to *C. longa* and particularly to its rhizomes. The chemical structure of curcumin was determined in the 1970s; the potential uses of curcuminoids in medicine have only been recently reported. The structure of curcumin as diferuloylmethane was confirmed with degradation work (*67*,*68*). Turmeric was found to be a rich source of phenolic compounds or curcuminoids. Curcumin was first isolated during 1815 (*69*). Besides curcumin, two minor constituents were also isolated (*70*). Later, many researchers optimized the extraction of these compounds by a variety of methods (*71*). Recently, Baumann et al. (*72*) efficiently extracted curcuminoids using supercritical CO_2 modified by 10% ethanol. Although supercritical fluid extraction is considered to be a clean technology resulting in acceptable yields and purity, it possesses a disadvantage because it involves high operating pressures. The scale-up problems could also be severe when the extraction is possible in a larger scale. Park and Kim (*73*) reported two novel compounds, namely, 4″-(3‴-methoxy-4‴-hydroxyphenyl)-2″-oxo-3″-enebutanyl 3-(3′-methoxy-4′-hydroxyphenyl) propenoate (calebin-A) and 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,4,6-heptatrien-3-one, and seven known compounds, namely,

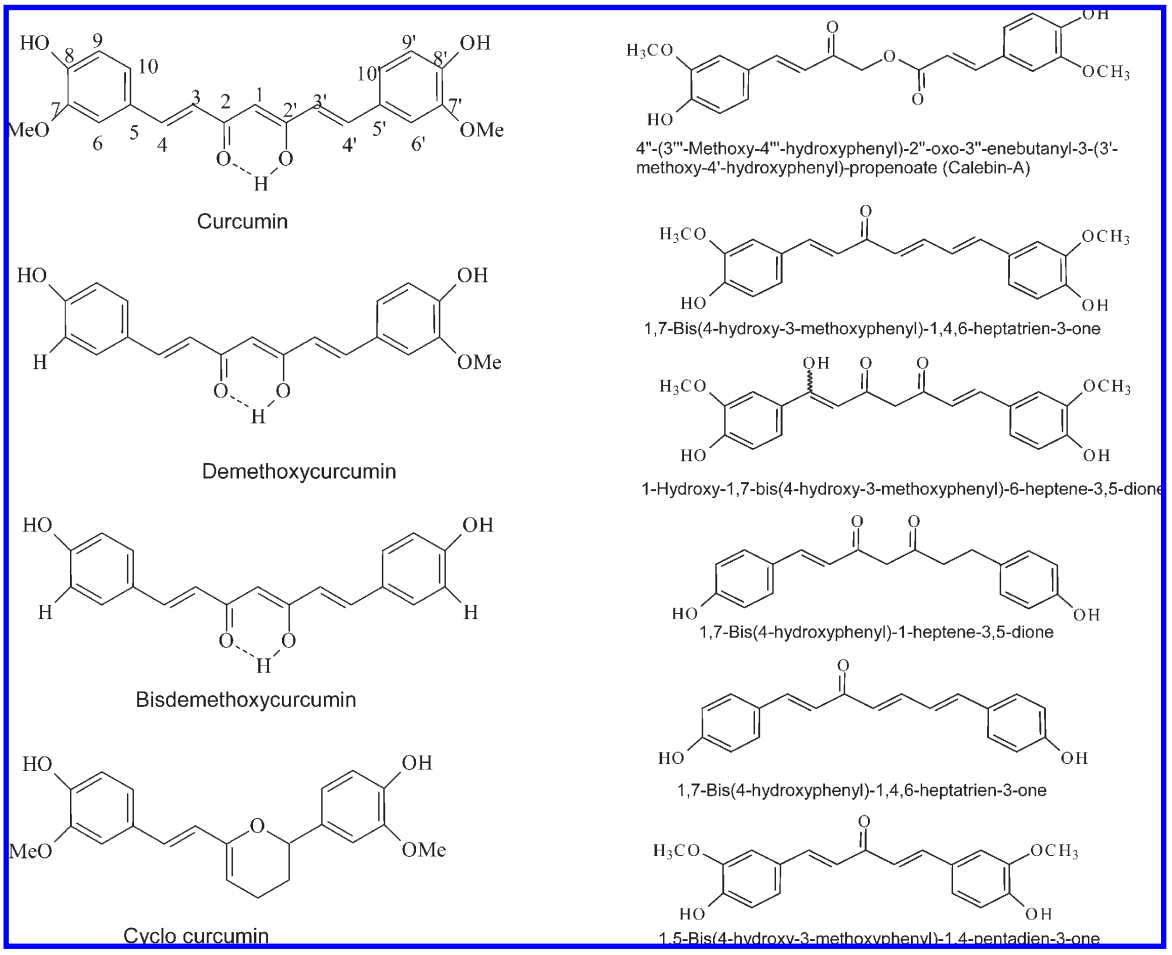

Figure 3. Structures of curcuminoids reported from turmeric.

curcumin, demethoxycurcumin, bisdemethoxycurcumin, 1-hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-6-heptene-3,5-dione, 1,7-bis(4-hydroxyphenyl)-1-heptene-3,5-dione, 1,7-bis(4-hydroxyphenyl)-1,4,6-heptatrien-3-one, and 1,5-bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one, from *C. longa* (**Figure 3**). Recently, Dandekar and Gaikar (*74*) reported the hydrotropy-based extraction method for selective extraction of curcuminoids from *C. longa*. Sodium cumene sulfonate was reported to be an efficient hydrotrope for the extraction of curcuminoids. Numerous methods were available for isolating curcuminoids from *C. longa*. Because the isolation of pure curcumin from plant material is tedious, there are extracts available in the market that contain a mixture of the three curcuminoids, that is, curcumin (75−81%), demethoxycurcumin (15−19%), and bisdemethoxycurcumin (2.2−6.6%). Except by the chromatographic routes, all other methods generally converge to curcuminoids with curcumin as the major constituent (*75*−*77*). Recently, we have developed a rapid method for the purification of three curcuminoids by flash chromatography (*78*). This method is efficient and provides multiple grams of curcuminoids in a relatively short time.

The first literature on the therapeutic use of curcumin for human health was published in *The Lancet* in 1937(*79*). Curcumin has been extensively studied for health benefits. Research during 1950−1990 was focused on antimicrobial, antioxidant, and anti-inflammatory activities, on the basis of the association of inflammation with cancer. Later, research was focused mainly on the involvement of curcumin in multiple pathways to prevent cancer (*80*, *81*). Major biological activities of curcumin demonstrated through in vitro as well as in vivo studies are summarized in **Table 6**. Moreover, recent literature substantiates various biological activities of curcumin. In addition, in vitro studies have demonstrated that curcumin may inhibit leucotriene formation, enhance wound healing (*66*), and act as a potent antioxidant (*82*). As of today, more than 8000 research papers are available on the biological activities of curcumin. During the past decade, a number of clinical studies have been reported on curcumin due to its anti-inflammatory potential.

Clinical Trials of Curcuminoids. On the basis of the highly promising results from both in vitro and in vivo experiments, curcumin has been evaluated for various health benefits in clinical trials. Some of the potential benefits clinically evaluated are inflammation, cholesterol-lowering effects, and cancer with emphasis on colon and pancreatic cancers. Curcumin has been subjected to clinical trials for anti-inflammatory, anticancer, and retroviral activities. Three of the clinical studies have demonstrated that curcumin is safe at a dose of 12 g/day. A clinical study conducted on young volunteers for antirheumatic activity has shown that curcumin was well tolerated and exhibited antirheumatic activity comparable to that of phenylbutazone (*83*). Other clinical studies of curcumin include skin cancer lesion, lowering of serum cholesterol, preventing formation of gallstones, chronic anterior uveitis and idiopathic inflammatory orbital pseudo tumors, psoriasis, colon carcinoma, irritable bowel syndrome, and benefits in advanced pancreatic carcinoma (*84*).

Cheng et al. (*85*) reported a phase I clinical trial of curcumin for 25 patients with high-risk or premalignant lesions. Curcumin was orally administered at a dose of 500 mg/day for 3 months, and the dose was simultaneously increased to 1000, 2000, 4000, 8000, and

Table 6. Major Biological Activities Reported for the First Time Using Curcuminoids

year	major research findings	refs
3000 B.C.	discovered by Indian system of medicine as potent health beneficial rhizome	*215* and refs cited therein
1280 A.D.	identified as a wonder root for health benefits by Marco Polo	*81* and refs cited therein
1815	curcumin isolated and characterized by Vogel and Pelletier	*216* and refs cited therein
1973	Roughley and Whiting determine the chemical structure	*217* and refs cited therein
1748	first recorded article referring to *Curcuma* spp., "de curcuma officinarum," published in 1748 by Loeber	*218* and refs cited therein
1937	first article referring to use of curcumin in human disease published in *The Lancet*	*79*
1941	nematocidal activity of curcuma using *Paramecium caudatum*	*219*
1972	anti-inflammatory and antiarthritic actions of volatile oil of *C. longa*	*220*
1982	anti-inflammatory effect of curcumin	*221*
1988	antitumor activity of curcumin demonstrated	*222*
1992	anti-venom activity against *Bothrops* and *Crotalus* venom	*223*
1992	inhibitory effects of curcumin on the proliferation of blood mononuclear cells and vascular smooth muscle cells	*224*
1992	curcumin as an inhibitor of leucotriene formation in rat peritoneal polymorphonuclear neutrophils (PMNL)	*225*
1994	lipid peroxidation inhibitory activity of curcumin	*226*
1995	antioxidative properties of curcumin and its three derivatives (demethoxycurcumin, bisdemethoxycurcumin, and diacetylcurcumin)	*227*
1995	curcumin has antiviral activity, being an HIV-1 integrase inhibitor	*228*
1998	wound healing activity of curcumin demonstrated	*66*
1998	antiprotozoal activity of curcumin against *Leishmania amazonensis*	*229*
1999	antimicrobial and antimutagenic components of turmeric identified	*230*−*232*
2000	curcumin shown to have cholesterol-lowering effect similar to that of statins	*233*
2000	inhibition of NFκB by curcumin demonstrated by Anto and co-workers	*234*
2006	antioxidant activities of curcumin, demethoxycurcumin, and bisdemethoxycurcumin demonstrated	*82*
2007	clinical study on pancreatic cancer in progress at M. D. Anderson Cancer Institute, USA	*235*

12000 mg/day. The concentration of curcumin in serum and urine was determined by HPLC. Interestingly, no toxicity was observed for up to 8000 mg/day. A recent study underscored the notion that a single high dose of curcumin (0.5−12 g) is safe in healthy human volunteers (*86*). Results of a randomized placebo-controlled double-blind study of curcumin (2 g/day for 6 months) in patients with ulcerative colitis seem to suggest that curcumin combined with sulphasalazine or mesalamine seems to prevent relapse (*75*, *87*). Pharmacokinetics studies in humans have shown that curcumin is detectable as glucuronide and sulfate conjugates in plasma, and administration of 10 and 12 g has shown plasma concentrations of 35.33 ± 3.78 and 26.57 ± 2.97 μg/mL, respectively, with a ratio of glucuronide to sulfate of 1.92:1 (*76*). Similarly, in another pilot study involving 10 patients with ulcerative colitis or Crohn′s disease, curcumin at 1.11 and 1.65 g daily for 2 or 3 months appeared to delay progression of disease. However, interpretation of results from such a small number of trial participants is difficult (*77*). Pilot studies of curcumin at dose levels up to 3.6 g per day in cancer patients conducted at Leicester University suggest that curcumin is safe and may reduce oxidative DNA adduct levels in gastrointestinal target tissue (*88*), but not in liver (*89*). In another study, administration of 3.6 g of curcumin for 4 months demonstrated a decrease in colon cancer by reducing prostaglandin E2 by 60% (*89*). Currently, a phase III clinical trial is in progress to treat pancreatic cancer using a combination of gemcitabine and curcumin in the United States and other countries.

Curcumin did not gain significant attention by cancer researchers due to its antimicrobial and anti-inflammatory activity until after 1990. This could be due to understanding inflammation as key player in most cancers and due to its effect on multiple signaling pathways and its ability to target several biochemical pathways, which are known to alter during the development of cancer. Curcumin is capable of blocking the transformation, proliferation, and invasion of tumor cells. It is also known for suppressing several tumor cell lines including drug resistance ones (*90*). This unique potential of curcumin has made it a more successful candidate as a chemopreventive agent in colon and pancreatic cancer as evident from results based on ongoing clinical studies (*91*, *92*). As observed with most of the natural compounds, bioavailability is one of the limiting factors for complete utilization of curcuminoids. Curcumin is known for its very poor bioavailability (< 0.01%), due to destruction from enzymes such as UGT (UDP-glucuronosyltransferase), sulfotransferase, alcohol dehydrogenase, and p450. Some studies seem to demonstrate that natural compounds, such as piperin, quercetin, and genistein, may inhibit these enzymes, which can enhance bioavailability (*93*, *94*). It is critical that studies use pharmaceutical approaches to enhance bioavailability through novel delivery strategies such as nanoparticles, liposomes, and defined phospholipid complexes. Research efforts are necessary to enhance the bioavailability of such a potent molecule either through biotechnological or pharmaceutical routes; otherwise, mankind will fail in utilizing the maximum potency of such a wonder molecule.

ASCORBIC ACID

Discovery and Chemistry. Ascorbic acid was discovered after searching for centuries to cure scurvy, and the name was coined from "antiscurvy" due to its dramatic cure of the condition. A Scottish naval surgeon named James Lind discovered the ability of ascorbic acid to treat many soldiers and published his finding as *Treatise on the Scurvy* in 1753. On the basis of Lind's observation, lime juice was prescribed to British navy soldiers for treatment of scurvy in 1795. In 1907 Axel Holst and Theodore Frolich, two Norwegian biochemists, showed that withdrawal of ascorbic acid containing food led to scurvy and the same is reversed upon feeding with food rich in ascorbic acid. Furthermore, between 1910 and 1920 Zilva and another scientist attempted to isolate ascorbic acid from various food sources (*95*, *96*). Sir Walter Norman Haworth, the English chemist, determined the chemical structure of ascorbic acid for which he was awarded the Nobel Prize in 1937 (*97*). Stereoisomers of ascorbic acid are shown in **Figure 4**. Ascorbic acid was first synthesized in 1933 by the Polish−Swiss endocrinologist Tadeus Reichstein (*98*), whereas the first report of ascorbic acid synthesis from a noncarbohydrate precursor was reported in 1998 by Banwell and co-workers from Australia. The authors used

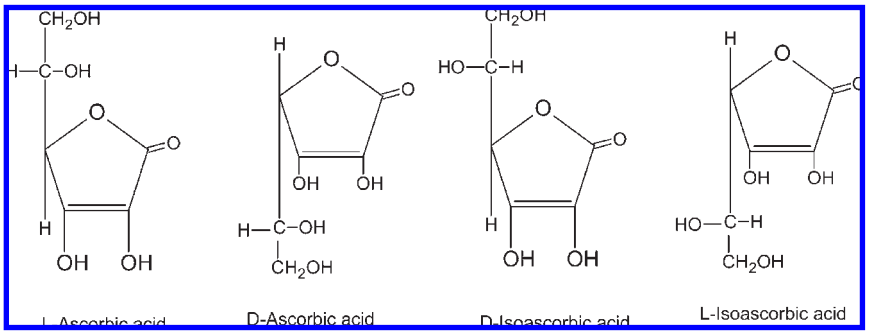

Figure 4. Structures of stereoisomers of ascorbic acid.

chlorobenzene as a starting material for the synthesis of ascorbic acid and major intermediates were *cis*-1,2-dihydrocatechol 2 and 3,5-*O*-benzylidene-L-gulonolactone (*99*). The first report on the oxidation reduction potential of ascorbic acid was published in 1933 by Eric G. Ball of Johns Hopkins University (*100*). Production of *d*-araboascorbic acid from *Penicillium* was reported in 1960 (*101*). Information regarding the biosynthesis of ascorbic acid in animals except human, primates, guinea pigs, birds, and fishes was reported by Chatterjee (*102*).

Several methods were developed for the analysis of ascorbic acid and dehydroascorbic acid. Unfortunately, most of these methods are lacking specificity toward the measurement of actual levels of ascorbic acid and dehydroascorbic acid in the samples. Spectrophotometric (*103*), titration (*104*), and enzymatic methods (*105*) were reported for the accurate analysis of ascorbic acid and dehydroascorbic acid. However, these methods were not practical due to the interference of other compounds in biological samples. HPLC with UV detection is the most common tool for the accurate analysis of ascorbic acid (*106*). Moreover, the stability of ascorbic acid and dehydroascorbic acid is very important because they are not stable at high temperature, and they are sensitive to changes in pH, light, and metal ions. At neutral pH dehydroascorbic acid has a half-life of 2 min (*107*), whereas ascorbic acid has a pK_a value of 4.2 (*108*). Hence, the pH of the extraction solvent plays a key role in maintaining the stability of the ascorbic acid and dehyroascorbic acid. Trichloroacetic acid and metaphosphoric acid were commonly used for the extraction of ascorbic acid from samples (*109*, *110*).

Although some methods were reported for the simultaneous determination of ascorbic acid and dehyroascorbic acid (*111*), this may not be possible due to low absorption of dehyroascorbic acid in UV. Conversion of dehyroascorbic acid to ascorbic acid by derivatizing agents is another common approach for the quantification of total ascorbic acid (*112*). In precolumn derivatization, dehydroascorbic acid is transformed into a fluorescence compound that has more selective absorption in the UV range.

Biological Activities of Ascorbic Acid. Discovery of involvement in the synthesis of collagen was one of the hallmarks of ascorbic acid research. One of the initial understandings in this direction is the involvement of ascorbic acid in the conversion of pro-collagen to collagen. Ascorbic acid is required for the conversion of proline to hydroxyproline, which is essential for collagen synthesis. It seems that these studies were based on experiments conducted using ascorbic acid deficit conditions. Furthermore, Mitoma and Smith demonstrated using scorbutic conditions that the incorporation into collagen of both proline and hydroxyproline is decreased and that the hydroxylation of proline was found to be unaffected (*113*). Later research using cultured fibroblasts demonstrated the multifaceted role of ascorbic acid in the synthesis of collagen. Some of these include post-transcriptional hydroxylation and peroxidation (*114*).

Ascorbic acid is known for a number of vital biological activities including synthesis of collagen, neurotransmitters, steroid hormones, and carnitine, responsible for the conversion of cholesterol to bile acid. Apart from this, it is also known to enhance the bioavailability of iron, promote calcium absorption, and help in the healing of wounds and burns. Ascorbic acid is essential for healthy gums and prevention of blood clotting. Apart from antiscurvy, other major clinical investigations were conducted to understand the benefits in prevention of the common cold, iron absorption, colorectal carcinoma, ulcers, hypertension, prevention of atherosclerosis, and advanced malignancy (*115*). Outcomes of selected clinical investigations are summarized in **Table 7**.

Ascorbic acid when fed at 2 g/day has been demonstrated to reverse vascular endothelial vasomotor dysfunction in bronchial circulation of patients with coronary heart disease (*116*). One possible reason for this activity is through the effect of ascorbic acid on endothelium-derived vascular relaxing factor (EDRF) by scavenging superoxide, which is known to limit the activity of EDRF after reaction with nitric oxide (*117*). Ascorbic acid can also regulate activity of EDRF by depleting the number of reduced thiols, which in turn reduce NO production. Ascorbic acid is known to reduce oxidation of LDL by preventing aqueous oxidants and modification of LDL by dehydroascorbic acid, which make LDL resistant to metal ion induced oxidation (*118*). Ascorbic acid from dietary source has also shown protection of human sperm against oxidative DNA damage, which could lead to abnormalities of sperm leading to genetic defects (*119*). In addition, ascorbic acid is also useful in diabetics; ascorbic acid has been shown to reduce blood pressure (average reduction of 10 mmHg) and arterial stiffness in type II diabetes individuals (*120*). The possible mechanism of action is through enhanced endothelial nitric oxide synthase activity, which is either due to regulation of the redox state or an increase in the intracellular content of tetrahydrobiopterin (*121*). Another explanation is the possible reduction of insulin resistance by ascorbic acid, because insulin is known to cause endothelium-dependent, nitric oxide-mediated vasodilatation, by increasing insulin sensitivity to ascorbic acid (*122*). There are a number of clinical research reports on the association of a higher risk of ischemic heart disease in individuals with lower plasma ascorbic acid content (*123*).

The role of ascorbic acid in the prevention of cancer has been studied extensively; initial research suggested that the ability of ascorbic acid in reduction of cancer was associated with host resistance to neoplastic growth. One of the potential roles of ascorbic acid in the prevention of cancer is to maintain intracellular matrix integrity, which can prevent cancer through the following processes: infiltration of malignant, selective restriction of nutrition to tumor, protecting pre-existing collagen barriers for neoplastic erosion, and facilitating protective collagen encapsulation to healthy tissues (*124*). On the other hand, feeding

Table 7. Major Clinical Studies Conducted Using Ascorbic Acid for Health Benefits

year published	trial conducted	major outcome of the trial	ref
1974	ascorbic acid supplementation in the treatment of pressure sores	ascorbic acid may accelerate the healing of pressure sores	*236*
1975	effect of ascorbic acid in common cold	no major benefits of ascorbic acid observed in common cold	*237*
1982	randomized trial of ascorbic acid in *Polyposis coli*	ascorbic acid can influence polyp growth or turnover	*238*
1990	effect of ascorbic acid on absorption of iron	ascorbic acid can help in absorption of iron	*239*
1994	ascorbic acid as one of the vitamins to prevent colorectal carcinoma	more than vitamins C and E their dietary factors may help in prevention of colorectal carcinoma	*240*
1995	randomized clinical trial of ascorbic acid in the treatment of pressure ulcers	data did not support benefits of ascorbic acid in peptic ulcer	*241*
1995	treatment of hypertension with ascorbic acid	ascorbic acid can help in hypertension	*242*
2001	ascorbic acid as one of the vitamin for benefits in prevention of atherosclerosis	>74% decrease in incidence of atherosclerosis in vitamin-fed subjects	*243*
2004	prevention of nephropathy in renal dysfunction undergoing coronary angiography or intervention	ascorbic acid beneficial in prevention of nephropathy	*244*
2008	phase I clinical trial of intravenous ascorbic acid in advanced malignancy	high dose of ascorbic acid well tolerated and failed to demonstrate anticancer activity when administered to patients with previously treated advanced malignancies	*148*

an excess of ascorbic acid (10 g/day) has not provided benefits in the prevention of cancer. Interestingly, feeding of 500 mg of ascorbic acid twice a day for two weeks led to enhanced plasma concentration of ascorbic acid, which seems to protect against gastric cancer. One of the speculated mechanisms of protection is through reduction of *Helicobacter pylori* levels, which is a major cause for gastric cancer (*125*). Another research study has concluded that the gastric cancer prevention ability of ascorbic acid is through scavenging of oxygen radicals (ascorbyl) in the mucosal layer (*126*). A review publishing data based on epidemiological evidence seems to explain benefits of ascorbic acid in the prevention of cancer. For example, of 46 studies conducted, 33 studies have shown statistically significant protection against cancer. In another study, of 29 vitamin C-rich food supplements, 21 studies have shown significant protection against cancer, suggesting the role of ascorbic acid through radical scavenging or through other nutrition-related mechanisms (*127*). In contrast to this study as mentioned before, feeding an excess of ascorbic acid (10 g/day) has not provided benefits in the prevention of cancer (*128*, *129*). Overall, there are a greater number of research studies explaining beneficial effects of ascorbic acid in various cancers. It is clear that the supplements provided in the diet or as pharmaceutical formulations may not be beneficial once the cancer is initiated. These results indicate that ascorbic acid may serve as a preventive by avoiding the onset of the disease at the initiation stage of the disease.

CITRUS LIMONOIDS

Chemistry. Limonin, a triterpenoid, was first identified as the bitter constituent of citrus seeds in 1841 by Bernay (*130*). Despite its identification, not much progress was made until the 20th century. Higby in 1938 reported the isolation of limonin and isolimonin from Valencia and Navel orange and investigated some of the physical properties (*131*). In the meantime investigators isolated similar dilactone compounds from amur cork tree (*Phillodendron amurense* Rupr.); however, Schechter in 1940 showed that these dilactones, known as obaculactone and evodin dictamnolactone, are essentially limonin (*132*). Several other limonoids such as obacunone, obacunoic acid, isolimonic acid, and dictomnolide were identified around the same time (*133*). A major focus of early investigations was determination of the structure of the limonin and related bitter compounds in citrus juice. It has been established that limonin and related compounds are dilactones (*134*). The lactone group was found to be very stable to mild alkali and acid treatments (*135*). Nomilin, another bitter limonoid, was isolated from orange and lemon seeds, and the molecular formula was established as $C_{26}H_{32}O_8$ (*136*). Obacunone was isolated from citrus seeds using isopropyl alcohol (*137*). The most important milestones in limonoid chemistry are summarized in **Table 8**. A major focus of early research was on elucidation of the chemistry and structure of limonin (*138*). Limonin was found to contain two lactone rings with the molecular formula of $C_{26}H_{30}O_8$, which can be opened reversibly, a β-substituted furan ring, a ketonic oxygen atom, and two ethereal oxygen rings (*139*). Later, the triterpenoid nature of the limonin structure was established (*140*, *141*). Arnott et al. (*142*) published the first molecular structure of limonin based on X-ray crystallography. At the same time a research group in Japan investigated the structure of obacunone and showed the presence of three double bonds, a furan ring, and α,β-unsaturated lactone (*143*). Moreover, alkali treatment of obacunone led to the formation of obacunoic acid and isoobacunoic acid (*144*).

Another major facet of early limonoid research was the identification of new molecules. **Figure 5** depicts some of the structures of citrus aglycones and glucosides of limonoids.

Dreyer (*130*, *144*, *145*) purified and identified several new limonoids from various citrus species such as deacetylnomilin, deoxylimonin (*146*), rutaevin (*145*), and ichangin (*130*). NMR spectroscopy was first applied for limonin structural determination in 1960 (*142*); however, extensive use of NMR for elucidation of limonoid structures began in 1965 (*144*). Limonin was also shown to be the principal component of grapefruit juice bitterness along with naringin (*133*). Limonin was later identified as the major compound responsible for bitterness in several citrus juices. This observation led to an increased interest in the identification of its precursor, which was identified as limonin monolactone (*133*, *134*). However, a natural debittering process, that is, glucosidation of bitter aglycone, occurs during maturation of the fruit. The glucosides of limonoid were first identified in neem tree (*Melia azedarach*) (*135*) and later reported from the *Citrus* spp. (*136*). Until now, 39 limonoid aglycones and 17 limonoid glucosides have been isolated and identified from different species of citrus (*147*). Previously, citrus limonoids were considered a major problem for the citrus juice industry, as they cause delayed bitterness of the juices at room temperature, thus lowering the quality and value of the commercial juice. However, at present, these bitter compounds have gained significant importance in the inhibition of several chronic diseases.

Analytical Methods. Developing analytical techniques to determine limonin and other limonoids in the juice and other

Table 8. Milestones in the Chemistry of Limonoid Research

year	achievement	refs
1841	identification of limonoids in citrus seeds by Bernay	*245* and refs cited therein
1938	isolation of limonin and isolimonin from orange seeds	*245*
1940	identification of limonin in different *Citrus* spp.	
1946	identification of obacunone, obacunoic acid, isolimonic acid	*246*
1946	structure of limonin established	
	lactone group shown to be very stable	*146*, *247*
1948	nomilin isolated from orange and lemon seeds; formula established as $C_{26}H_{32}O_8$	*248*
1951	obacunone isolated from citrus seeds	*137*
1951	structure of limonin molecule established chemical modifications	*249*
1960	limonin found to contain two lactone rings that can be opened reversibly, a β-substituted furan ring, a ketonic oxygen, and two ethereal oxygen rings	*142*
1960	structure of limonin established using X-ray crystallographic techniques	*142*
1961	presence of three double bonds, a furan ring, and α,β-unsaturated lactone in obacunone	*143*
1961	formation of obacunoic acid and isoobacunoic acid by alkali treatment of obacunone	*143*
1960	application of NMR spectroscopy	*131*
1965	extensive application of NMR for limonoid structure elucidation	*132*
1965	limonin shown to be principal component of grapefruit juice	*133*
1965−1967	purification of deacetylnomilin, deoxylimonin, rutaevin, and ichangin from *Citrus* spp.	*130*, *145*, *146*
1968	identification of limonin monolactone, precursor of limonin	*134*
1970	development of thin layer chromatographic technique for limonoid identification	*138*
1975	development of HPLC techniques for limonoid quantification	*139*
1986	glucosides of limonoids reported from *Melia azedarach*	*135*
1989	limonoid glucosides were reported from *Citrus* spp.	
2000	development of liquid chromatography coupled with mass spectroscopy for citrus limonoid analysis	*136*
2002	preparative level purification of limonin glucoside	*151*
2006	improved method of large-scale limonoid purification	*152*

fruit parts became imperative and led to the development of thin layer chromatography for limonoids (*138*). Subsequently, development of high-performance liquid chromatography (HPLC) provided a useful and versatile tool for the quantification and identification of limonoids. The HPLC method was first used for quantification of limonin (*20*). The quantitative methods using HPLC have improved since their inception by including a higher number of compounds in normal phase and reverse phase (*148*). More recently, a procedure for the simultaneous determination of limonoid aglycones and glucosides has been published (*139*, *149*). With the advent of mass spectrometric techniques coupled with liquid chromatography the resolution and precision of HPLC has improved in recent years. Several analytical methods have been reported over the years involving HPLC coupled with different versions of mass spectrometry (*150*).

In recent years, a tremendous amount of interest has been developed in citrus limonoids owing to their anticancer activity. A major challenge for the study of the biological activity of limonoids is their purification in large quantities. Usually purification of limonoids is a complex procedure and involves several steps. Preparative level purification of limonoid glucosides has been reported but involves several steps (*151*). Recently, an improved method for large-scale purification of limonoids has been reported (*152*).

Biological Activity. Currently, limonoids have been gaining attention due to their wide range of biological activities. The research in our laboratory and other groups has shown that citrus limonoids are found to possesses various biological activities, especially anticancer activity (*153*). Major impacts on biological activity of limonoids from researchers have been summarized in **Table 9**. Pettit et al. studied the structure−activity relationship of limonoids from the order Rutales including citrus limonoids (*154*, *155*). Limonin and nomilin were found to inhibit forestomach neoplasia in a mouse model (*156*), possibly by inducing GST enzyme in the small intestine mucosa (*157*). Limonoids have shown inhibition of colon cancer, ovarian cancer, and human neuroblastoma cells in in vitro studies (*158*) and inhibit the growth of estrogen receptor-negative and -positive human breast cancer cells (*159*). Some limonoids have also been implicated in the inhibition of the development of oral tumors in a hamster cheek pouch model (*160*). Limonin and nomilin have been found to inhibit p-24 antigen activity, protease activity, and replication of HIV-1 (*161*). Additionally, limonoids have been shown to reduce LDL cholesterol levels in a rabbit model and apo-B production in HepG2 cells (*162*). Furthermore, limonoids have been shown to possess antifeedant activity against insects (*163*).

FUTURE PERSPECTIVES AND CHALLENGES

Bioactive compounds or secondary metabolites are essential components of plants (**Figure 6**). Understanding the role of these bioactive compounds in the maintenance of health is beginning to emerge recently, even though the existence of these compounds has been known for long time. The majority of studies involving bioactive compounds followed a degradative approach, that is, studying one or a few compounds at a time. However, the overall effect of a particular fruit or vegetable cannot be accounted for by one or a few components present therein. Therefore, strategies looking into the synergistic effect of these components, specifically the food matrix, are more appropriate. Although several studies such as the Rotterdam Study and the Zutphen Elderly Study (*33*, *164*) investigated the effect of bioactive compounds, the information about the bioactive compounds intake was derived from the recorded dietary habits. The bioactive compounds and/or their combination in a specific diet were not provided against a particular chronic condition. Therefore, current data lack information tailored to delivery of bioactive compounds. On the other hand, several studies have looked into the effect of the consumption of a particular fruit or vegetable against certain chronic conditions. This approach also lacks basic information about the bioactive compound profile of the fruit or vegetable. Using this type of study, often the choices were made on the basis of one or a few bioactive compounds without taking into account the possible synergistic or contradicting effect of

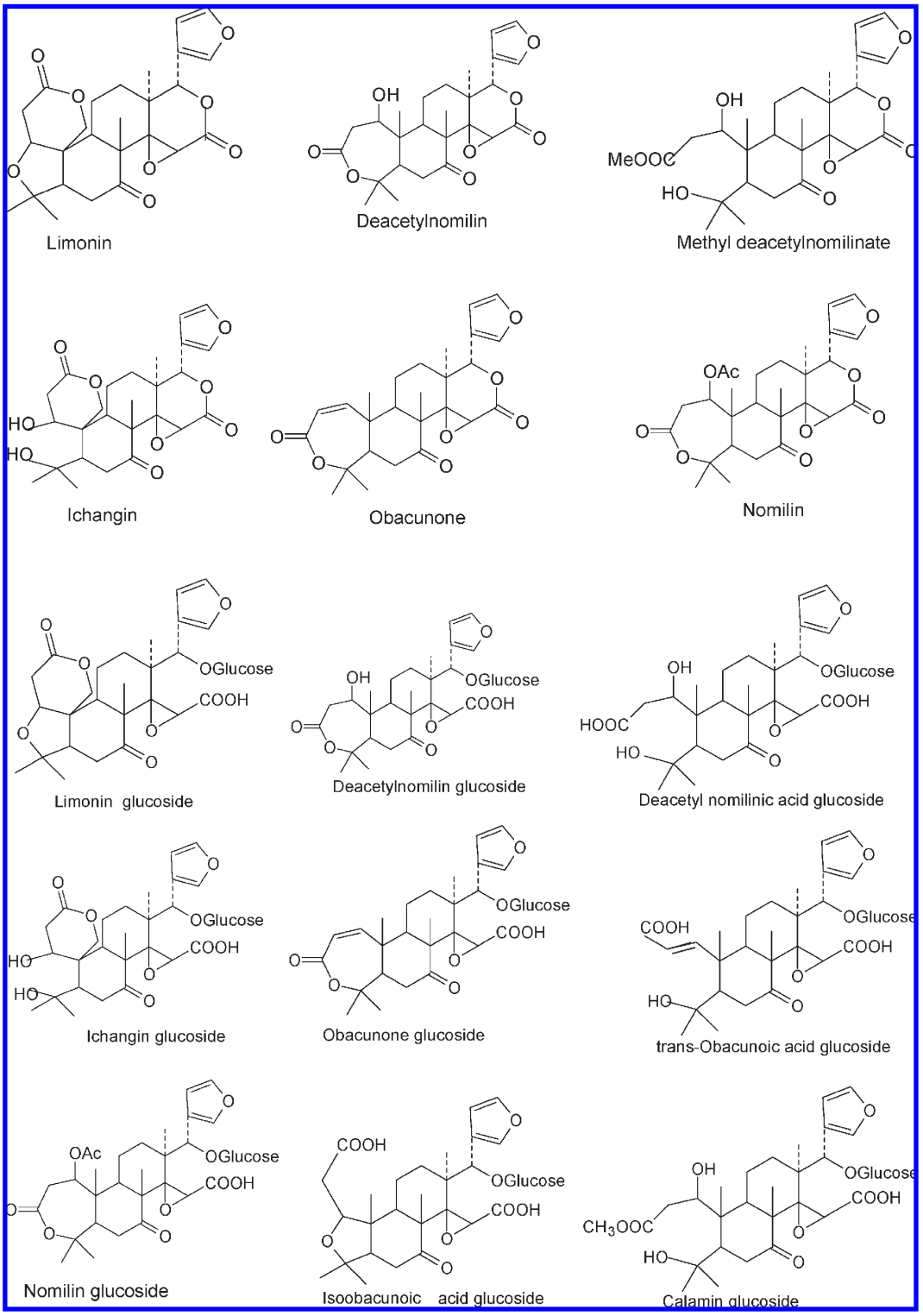

Figure 5. Structures of limonoids commonly found in citrus juice.

different bioactive compounds. For example, accumulating literature related to anticancer activity of various bioactive compounds suggests a possible chemopreventive ability when studied using isolated individual compounds. However epidemiological and cohort studies failed to find correlations between the consumption of fruits or vegetables rich in a particular component. Therefore, it is imperative to study the possible interaction of different bioactive compounds present in a fruit or vegetable matrix. Another major limiting area of study is the bioavailability of these components in the food matrix. Bioavailability is a major factor determining the overall effect of bioactive compounds, particularly from different food matrices. Absorption of different constituents can be very complex depending upon the food matrix consumed, and investigation in this aspect will provide a better understanding for development of a tailored approach. Certainly, this does not undermine the importance of further investigations using in vitro experiments, which are needed to establish an a priori case for the bioactive compounds. Therefore, there are several key areas of investigation that need to be pursued vigorously. Data generated from investigation will provide the answer for the some of the anomalies currently present in the literature.

Table 9. Milestones in Biological Activity of Limonoid Research

year	achievement	refs
1983	evaluation of citrus limonoids against leukemia cell line	*153*
1986	antifeedant activity of citrus limonoids	*163*
1989	inhibition of forestomach neoplasia in mice by citrus limonoids	*154*
1989	induction of GST by citrus limonoids	*155*
1989, 2004	inhibition of oral carcinogenesis by citrus limonoids	*157, 250*
1997	molt inhibition of mosquito larvae	*251*
2001	inhibition of breast cancer and ovarian cancer cells in vitro by citrus limonoids	*156*
2001	inhibition of colon carcinogenesis in vivo in rats	*252*
2003	anti-HIV activity	*161*
2000	regulation of apo-B production by citrus limonoids	*253*
2006	inhibition of colon carcinogenesis in mice	*254*
2006	inhibition of neuroblastoma cell line	*255, 256*
2008	inhibition of colon cancer cells (HT-29)	*147*

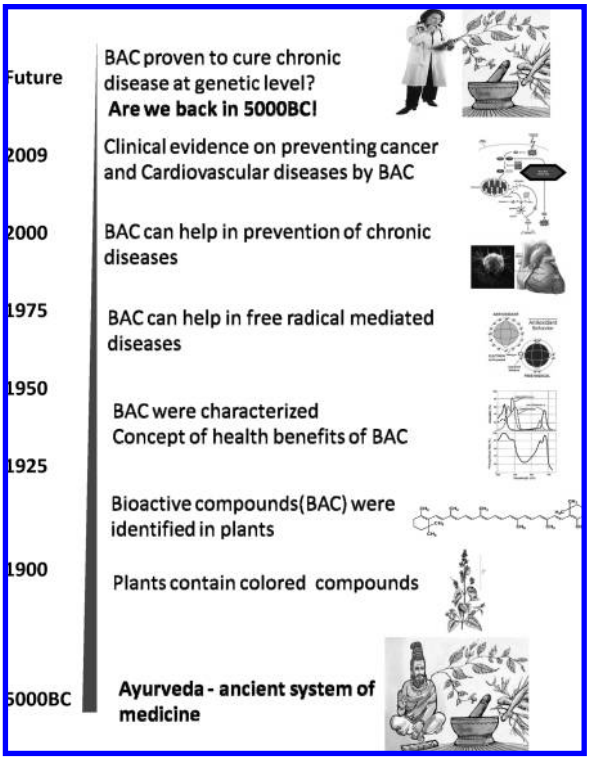

Figure 6. Journey of knowledge on bioactive molecules from ancient system to current research.

SUMMARY

Vegetables and fruits are rich sources of secondary metabolites or bioactive compounds. Numerous studies have provided evidence for the beneficial effects of these bioactive compounds and consumption of vegetablesand fruits. We have compiled information considering ancient knowledge (curcumin), abundance (flavonoids and ascorbic acid), and less explored compounds (citrus limoniods). The literature on these compounds has provided some of the vital information regarding potential health benefits. Bioactive compounds from vegetables and fruits have the potential to prevent certain chronic conditions such as cancer, cardiac diseases, and diabetes. Whereas several in vitro studies have demonstrated a possible prevention of cancer in clinical trials, the same was not observed in meta-analysis, probably because of the small number of studies considered for such analysis. These bioactive constituents are important due to their antioxidant activities and anti-inflammatory activities, altering the activity as well as levels of enzymes. Several bioactive compounds such as curcumin, lycopene, and β-carotene have been extensively studied and have the potential to improve human health through intervention strategies.

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