# *Theobroma cacao*: Review of the Extraction, Isolation, and Bioassay of Its Potential Anti-cancer Compounds

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**Abstract:** Plants have been a good source of therapeutic agents for thousands of years; an impressive number of modern drugs used for treating human disease are derived from natural sources. The *Theobroma cacao* tree, or cocoa, has recently garnered increasing attention and become the subject of research due to its antioxidant properties, which are related to potential anti-cancer effects. In the past few years, identifying and developing active compounds or extracts from the cocoa bean that might have anti-cancer effects have become an important area of health- and biomedicine-related research. This review provides an updated overview of *T. cacao* in terms of its potential anti-cancer compounds and their extraction, *in vitro* bioassay, purification, and identification. This article also discusses the advantages and disadvantages of the techniques described, and reviews the processes for future perspectives of analytical methods from the viewpoint of anti-cancer compound discovery.

**Keywords:** Natural Products, Anti-cancer, *Theobroma cacao*

## INTRODUCTION

Cancer is characterized by the proliferation of abnormal cells that fail to respond correctly to normal regulatory mechanisms. Carcinogenesis, which describes cancer development, is a multi-step process consisting of the initiation, promotion, and progression of uncontrolled cell growth (Bennett, Rojas, & Seefeldt, 2012; Plankar, Jerman, & Krašovec, 2011). Every year, there are 10 million new cancer cases and 7.6 million cancer-related deaths (13% of all deaths) worldwide; it has been estimated that there will be 12 million deaths in 2030 (Pujol et al., 2007; Talaviya, 2011). In Malaysia, a total 18,219 new cancer cases were diagnosed in 2007, involving 8,123 males (44.6%) and 10,096 females (55.4%). The 10 leading cancers among the Malaysian population are breast, colorectal, lung, nasopharynx, cervical, lymphoma, leukemia, ovarian, stomach, and liver cancer, and the five leading cancers among the general Malaysian population are cancer of the breast (18.1%), head and neck (13.2%); colorectum (12.3%), trachea, bronchus, and lung (10.2%), and cervix (4.6%) (National Cancer Registry, Malaysia, 2011).

Plants have been used for medicine for thousands of years, and remain relevant as natural resources of active compounds for treating human diseases, especially cancer (Cragg, Newman, & Snader, 1997; Cragg & Newman, 2013). As reported previously (Kinghorn et al., 1999; Kinghorn, 2000; Cragg & Newman, 2005), plant natural products play an important role among the many recent advances in cancer chemotherapy, having contributed considerably to the approximately 60 available cancer chemotherapeutic drugs. There are currently four structural classes of plant-derived anti-cancer agents on the market in the USA, namely, the *Catharanthus* (vinca) alkaloids (vinblastine, vincristine, vinorelbine), epipodophyllotoxins [etoposide, etopophos (etoposide phosphate), teniposide], taxanes (paclitaxel, docetaxel), and camptothecin derivatives (topotecan, irinotecan). Many current cancer research studies investigating the chemotherapeutic potential of medicinal plants have been carried out to discover new therapeutic agents or complementary alternative medicines that lack the toxic effects of chemotherapeutic drugs and have the same or better curative effects associated with the current synthetic therapeutic agents (Rao, Kumar, Islam, & Mansour, 2008; Mahavorasirikul, Viyanant, Chaijaroenkul, Itharat, & Na-Bangchang, 2010). In the early stage of discovery, plant-derived anti-cancer agents are evaluated by screening their activity, which is based on their antiproliferative effects. Such

potential anti-cancer compounds are also investigated to elucidate their mechanism of action against various cancer cells.

The tropical tree *Theobroma cacao*, or cacao or cocoa, belongs to the family Sterculiaceae (alternatively, Malvaceae *sensu lato*), order Malvales. The cocoa tree originated from ancient Central America, where the Mayans and Aztecs cultivated it for its seeds, which they used for extracting a drink called *chocolatl*, a precursor to the modern chocolate. The Olmecs and Mayans believed that cacao was of divine origin. *Theobroma* means “food” (from the Greek *broma*) of the gods (from the Greek *theo*)” and *cacao* is derived from the Aztec Nahuatl word *xocolatl*, from *xococ* (bitter) and *atl* (water). The *T. cacao* tree is a small evergreen tropical and subtropical tree that originates from neotropical rainforests, primarily in the Amazon basin and the Guyana Plateau (Bhattacharjee & Kumar, 2007; Zhang, Figueira, Motilal, Lachenaud, & Meinhardt, 2011; Bernaert, Blondeel, Allegaert, & Lohmueller, 2012). In Malaysia, the first cocoa tree (Figure 1) was planted in Malacca in 1778 (Ruzaidi, Amin, Nawalyah, Hamid, & Faizul, 2005). Cocoa was used by ancient peoples as a medicinal plant for treating various disorders. Over 100 medicinal uses for cocoa have been documented in Europe and New Spain from the 16th to early 20th century; it has been used to treat anemia, mental fatigue, tuberculosis, fever, gout, kidney stones, and even poor sexual appetite (Dillinger et al., 2000). The cocoa bean contains a large number of phytochemicals, and physiologically active compounds have been reported. For example, Kim, Lee, & Lee, 2011 reported that selected procyanidins present in cocoa inhibited tumorigenesis, tumor growth, and angiogenesis. Procyanidin-enriched cocoa seed extracts caused G2/M arrest and 70% growth inhibition in Caco-2 colon cancer cells. The consumption of cocoa or chocolate, which has high antioxidant activity, could be beneficial in decreasing damage caused by genotoxic and epigenetic carcinogens, and inhibit the complex processes leading to cancer.

The use of bioactive compounds from *T. caca*o in the pharmaceutical and food industries indicates a need for the most appropriate and standardized method for discovering such active components from plant materials. Ethnopharmacology-based phytochemical research is an effective approach for discovering novel active compounds that are potential anti-cancer drugs. The *T. cacao* parts traditionally used for treating disease represent a source of chemical entities, but little information is available on their nature, except for that of the cocoa bean (Arlorio et al., 2005; Maskarinec, 2009). Based

on this viewpoint, scientists need to determine the best methods for natural product research, which include the sample preparation, extraction, analytical techniques, bioassay guided fractionation, isolation, and characterization of plant bioactive metabolites as potential lead compounds in drug discovery. The analytical techniques employed are very important for purifying and identifying the active compound responsible for apoptotic activity, especially for agents that traditional use claims are anti-cancer. The efficiencies of conventional and nonconventional methods mostly depend on the critical input of parameters, understanding the nature of the plant matrix, and bioactive compound chemistry (Azmir et al., 2013; Brusotti, Cesari, Dentamaro, Caccialanza, & Massolini, 2014; Sasidharan, Chen, Saravanan, Sundram, & Latha, 2010). This article reviews the literature on the important steps involved in discovering anti-cancer agents in the cocoa bean, i.e., extraction, bioassay evaluation, purification and identification, and assay of the mechanism of action by the apoptosis pathway.

## POTENTIAL ANTI-CANCER COMPOUNDS IN COCOA

Interest in the potential health-related benefits of antioxidant- and phytochemical-rich dark chocolate and cocoa has increased, and many studies have reported the health benefits of the bioactive compounds (Table 1) and chemical compositions of cocoa and cocoa products. Much of the proposed health-protective activity associated with cocoa and chocolate consumption has been attributed to flavonoids, polyphenols, and procyanidins. The major reported pharmacological activity in cocoa includes antioxidant effects (Othman, Ismail, Abdul Ghani, & Adenan, 2007), protection against cardiovascular disease (Corti, Flammer, Hollenberg, & Lüscher, 2009), and anti-cancer effects (Maskarinec, 2009). Many *in vitro* or experimental model studies have shown that flavonoids have a wide range of biological activity related to anti-tumor effects, which includes the inhibition of several kinases and transcription factors (TFs) (Kang et al., 2008). Flavonoids appear to be antiproliferative, induce apoptosis, and inhibit angiogenesis; some are present in cocoa as monomers or procyanidins, and have been studied *in vitro*, and are most likely to be effective as anti-cancer agents (Eng et al., 2003; Faria, Calhau, de Freitas, & Mateus, 2006; Shoji et al., 2005; Zhao, Wang, Chen, & Agarwal, 1999).

Nuclear factor-κB (NF-κB) is a TF involved in inflammation, cell proliferation, and oncogenic processes (Sun & Karin, 2008). Activator protein-1 (AP-1) is a heterodimeric protein complex involved as a TF in apoptosis and cancer development, among other biological processes (Jeong, Kim, Hu, & Kong, 2004). *In vitro* studies on cocoa epicatechin, catechin, and quercetin, as well as procyanidin and B-type dimeric procyanidin extracts have shown that the compounds down-regulate NF-κB and AP-1 in cancer cell lines such as H–RS, Daudi, human monocytic THP-1, and Jurkat (Zhang et al., 2006; García-Mediavilla et al., 2007; Kang et al., 2008; Mackenzie, Adamo, Decker, & Oteiza, 2008). In contrast, the antioxidant effects of cocoa flavonoids delineate their putative beneficial action in controlling cell damage and tumor progression (Martin, Goya, & Ramos, 2013). The cocoa procyanidins, epicatechin, and catechin have powerful antioxidant effects (Belščak, Komes, Horžić, Ganić, & Karlović, 2009; Jonfia-Essien, West, Alderson, & Tucker, 2008; Miller et al., 2006).

Previously, it was shown that a naturally occurring, cocoa-derived pentameric procyanidin (pentamer) caused G0/G1 arrest in human breast cancer cells, and it was demonstrated that the pentamer selectively inhibited the proliferation of human breast cancer cells (MDA-MB-231, MDA-MB-436, MDA-MB-468, SKBR-3, MCF-7) and benzo(a)pyrene-immortalized 184A1N4 and 184B5 cells (Ramljak et al., 2005). The effect of cocoa powder and extracts with different amounts of flavonols and related procyanidin oligomers on human colonic cancer Caco-2 cell growth was also investigated: treatment with 50 mg/ml procyanidin-enriched (PE) extracts caused 70% growth inhibition with G2/M blockade, which might be an important target in the antiproliferative effects of cocoa polyphenols (Carnésecchi et al., 2002). The phytochemical compounds present in cocoa, especially flavonoids, are involved in various pathways and molecular targets for treating human cancer cells (Martin et al., 2013), evidence that cocoa can be considered a potential anti-cancer agent and that it needs to be developed for treating cancer.

## EXTRACTION OF COCOA ACTIVE COMPOUNDS

Plants contain a wide range of active compounds such as lipids, phytochemicals, pharmaceutics, flavors, fragrances, and pigments. Extraction is the first step in the utilization of such bioactive compounds, thus is used in virtually all investigations of natural plant compounds. Prior to extraction, fresh plant materials are typically rinsed with water, dried at room temperature, and ground to obtain a powder. The common method for extracting anti-cancer compounds from plants is solvent extraction. As stated by Dai & Mumper, 2010, solvent extractions are the most commonly used procedures for preparing extracts from plant materials due to their ease of use, efficiency, and wide applicability. Typically, the yield from chemical extraction depends on the solvent type, of which there are varying polarities, and extraction time and temperature, sample-to-solvent ratio, and sample chemical composition and physical characteristics. Anti-cancer agent solubility is governed by the chemical nature of the plant sample and the polarity of the solvent system used. The simplest extraction technique is solvent soaking (Owens, Straka, Carroll, & Taylor, 1998; Dai, Gupte, Gates, & Mumper, 2009). Generally, organic solvents such as methanol, ethanol, acetone, ethyl acetate, and combinations thereof, often with different proportions of water, are used for extracting anti-cancer compounds from plant materials. Thus, the organic solvent selected can affect the amount and rate of crude extracts.

Given its high phenolic compound content, especially procyanidins and flavonols, *T. cacao* is recognized as a major dietary source of antioxidants (Tomas-Barberan et al., 2007); three main polyphenol groups have been identified in cocoa: catechins or flavan-3-ols (37%), proanthocyanidins (58%), and anthocyanins (4%) (Belščak, Komes, Horžić, Ganić, & Karlović, 2009). Polyphenols have garnered much interest recently due to their antioxidant capacity and possible benefits to human health such as anti-carcinogenic, anti-atherogenic, anti-ulcer, antithrombotic, anti-inflammatory, immune modulating, anti-microbial, vasodilatory, and analgesic effects (Hii, Law, Suzannah, Misnawi, & Cloke, 2009). The extraction of phenolic compounds from plant materials is influenced by the chemical nature of the compounds, extraction method, sample size, time and storage conditions, and the presence of interfering substances such as proteins and carbohydrates (Garc´ıa-Ma´rquez et al., 2012; Koffi, Sea, Dodehe, & Soro, 2010). Yilmaz & Toledo, 2006 reported that, compared to a single-compound solvent

system, aqueous solutions of methanol, ethanol, and acetone dramatically improved polyphenol extraction. The relative recovery efficiency between solvents varies with different plant materials. The most commonly reported methods for polyphenol extraction are solvent maceration, such as Soxhlet extraction (Figure 2), hot water extraction, alkaline extraction, resin-based extraction, enzyme-assisted extraction, gamma- and electron beam irradiation–based extraction, and supercritical fluid extraction. However, some of these methods can cause the loss of bioactive compounds due to the high temperatures used and long extraction times; irradiation can present a health risk if proper care is not taken (Jin Dai & Mumper, 2010; Liu, Wu, Weng, & Tseng, 2005).

Extraction and product recovery are the most crucial steps in evaluating target molecules from various plant parts (Fulzele & Satdive, 2005). Presently, there is increasing demand for new extraction techniques with shorter extraction times, reduced organic solvent consumption, and increased pollution prevention (Wang & Weller, 2006). Currently, several extraction methods such as sonication-, microwave-, and ultrasound-assisted extraction, supercritical fluid extraction, accelerated solvent extraction, pressurized solvent extraction, and pressurized liquid extraction can be applied for recovering polyphenols (Wang & Weller, 2006; Wijngaard, Hossain, Rai, & Brunton, 2012). Quiroz-Reyes, Aguilar-Méndez, Ramírez-Ortíz, & Ronquillo-De Jesus, 2013 reported that ultrasonic radiation extraction yielded higher polyphenol content from both the husk and cotyledon of *T. cacao*, with high antioxidant activity. The shortcomings of the more established extraction methods have led to the use of new, sustainable, and innovative “green” techniques, e.g., microwave- and ultrasound-assisted extraction, that increase extraction efficiency, reduce time- and energy-consuming procedures, and contribute to environmental preservation by reducing the use of water and solvents, fossil energy, and the generation of hazardous substances (Chemat, Zill-e-Huma, & Khan, 2011). Lastly, the extraction process should provide the maximum yield of crude extract containing potential anti-cancer compounds, and of the highest quality (target compound concentration and potential anti-cancer activity of the extracts).

**ACTIVITIES OF BIOASSAY GUIDED COMPOUNDS**

The bioassay is a very important aspect in screening the anti-cancer activity of *T. cacao* plant part extracts, fractions, and pure compounds. The bioassay method used for other plants is compatible for use with the cocoa plant. Duarte, Rocha-Santos, Freitas, & Duarte, 2012 reported that active compound bioactivity establishes the potential application of natural products, so the design of the bioassay used is a crucial step in determining extract bioactivity for discovering target compounds based on said biological activity. It is essential that screening systems for natural products comprise a broad range of bioassays for exploring all possible anti-cancer activity. In bioassay screening, extract, fraction, and pure compound bioactivity can be screened *in vitro* and/or *in vivo*. *In vitro* testing can be performed at cellular or molecular level. In this review, the discussed bioassay method for evaluating the anti-cancer activity of cocoa plant parts is the 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Figure 3).

The MTT assay was developed by Mosmann (1983) as a semi-automated colorimetric assay for rapid assessment of proliferation and cytotoxicity in cell cultures. It is based on mitochondrial reduction of the tetrazolium salt MTT to formazan, which can be quantified spectrophotometrically (Nikkhah et al., 1992). The MTT assay is one of the most convenient assays for estimating the viability of animal or bacterial cells and offers great advantages over conventional assays such as hemocytometer counting (Freimoser, Grundschober, Aebi, & Tuor, 2000). MTT is a water-soluble yellow dye and can be reduced to water-insoluble purple formazan crystals by the dehydrogenase system of viable cells. Thus, the concentration of dissolved formazan crystals can be quantified spectrophotometrically and is directly proportional to the number of metabolically active cells (Wang, Wang, Tao, & Cheng, 2012). Dimethyl sulfoxide (DMSO) is considered one of the most suitable solvents for dissolution due to its high solubilizing efficiency and low volatility (Wang, Cheng, Wang, Wei, & Wang, 2010). The MTT assay is also widely used to determine the cytotoxicity, effect on proliferation, and activity of potential medicinal agents based on cellular mitochondrial dehydrogenase activity. As an example, a study of the effects of methanolic non-edible plant extracts of *T. cacao* on several cancer cell lines showed that the MTT assay could be applied with confidence to evaluate the antiproliferative activity of the extracts (Zainal, Abdah, Taufiq-Yap, Roslida, & Rosmin, 2014). Moreover, cocoa polyphenol-derived flavonols and procyanidins

exhibit antioxidant and anti-tumor properties. Previous studies also reported using the MTT assay to investigate the cellular growth inhibitory effect of chemically-synthesized procyanidin [3-O-galloyl]-(-)-epicatechin-(4β,8)-(+)-catechin-3-O-gallate (GECGC) in a variety of human cancer cell lines (Kim et al., 2008), the effect of cocoa extract polyphenols on MCF-7 cell viability (Oleaga et al., 2012), and the protective effects of a cocoa procyanidin fraction and procyanidin B2 (epicatechin-(4-8)-epicatechin) as the major cocoa polyphenol against hydrogen peroxide–induced apoptosis of rat pheochromocytoma PC12 cells (Cho, Lee, & Lee, 2008). These studies prove that the MTT assay is widely used in cell culture work involving cocoa extracts because it is simple, rapid, inexpensive, sensitive, accurate, reproducible, requires little material for detection, and can be employed as an initial screening approach for plant extracts (Muraina, Suleiman, & Eloff, 2009).

The MTT assay can be modified to screen for anti-cancer activity in all plant extracts. Briefly, cancer cells ($10^4$–$10^5$/well in 100 µl culture medium) are seeded in 96-well plates and treated with plant extracts for 24 h at 37°C in 5% $CO_2$. Typically, the treated cancer cells are incubated with MTT solution (20 µl, 5 mg/ml) at 37°C for 3–4 h, and lysed with DMSO. The yellow MTT is reduced by succinic dehydrogenase in the mitochondria of viable cells to purple formazan crystals, and the absorbance (OD) is measured at 570 nm using a microplate reader. The results are recorded as the $IC_{50}$, which is the sample concentration that inhibits 50% of cancer cell growth (Ibrahim et al., 2011; Mahavorasirikul et al., 2010; Sathiya & Muthuchelian, 2010). As mentioned by Atjanasuppat et al., 2009, anti-cancer activity can be categorized according to the $IC_{50}$ into four groups: active (≤20 g/ml), moderately active (>20–100 g/ml), weakly active (>100–1000 g/ml), and inactive (>1000 g/ml). The MTT assay has also been used to evaluate the anti-cancer activity of other plant extracts, such as *Centaurea jacea* L. against HeLa, MCF-7, and A431 cells (Forgo et al., 2012), *Typhonium flagelliforme* (Araceae) against NCI-H23 and HS578T cells (Lai, Mas, Nair, Mansor, & Navaratnam, 2010), *Platycodon grandiflorum* A. against HT-29, HRT-18, and HepG2 cells (Lee, Hwang, & Lim, 2004), South African plants against MCF-7, TK10, and UACC62 cells (Fouche et al., 2008), and *Tinospora crispa* (Batawali) against MCF-7, MDA-MB-231, and HeLa cells (Ibrahim et al., 2011). However, the assay cannot distinguish between cytostatic and cytocidal effects, does not quantify individual cell numbers, and is least efficient when performed in a medium that has

supported cellular growth for several days (Supino, 1995). Other than the MTT assay, there are few tetrazolium salt reduction assays for measuring viable cell numbers.

Mosmann, 1983 reported that the most frequently used tetrazolium salts are 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), and 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate water-soluble tetrazolium salt (WST-1). Despite their wide use and applicability, researchers using these assays may encounter compatibility issues depending on the study objectives. For example, compared to XTT and MTS, WST-1 is more stable, has a broader linear range, and enables more rapid color development. MTT can be reduced by nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FADH), and Nicotinamide adenine dinucleotide (NADH), but not by cytochromes. Tetrazolium salts are generally cytotoxic because the formazan crystals produced from the salt reduction must be solubilized with DMSO or hydrochloric acid/isopropanol, which destroys the cells being studied; therefore, time course experiments cannot be performed. However, there are other assays for determining cell viability, such as the intracellular adenosine triphosphate (ATP)-based, deoxynucleic acid (DNA)-based, and Alamar Blue assays. The ATP-based method is highly sensitive, reproducible, and simple for evaluating cell viability and proliferation. The amount of ATP present in the cells is proportional to the number of viable cells in culture. Similar results can be obtained using the DNA-based method, which is based on the measurement of cellular DNA to indicate the relative cell number, as cellular DNA content is highly regulated (Crouch, Kozlowski, Slater, & Fletcher, 1993; Jones, Gray, Yue, Haugland, & Singer, 2001; Mueller, Kassack, & Wiese, 2004; P. Wang, Henning, & Heber, 2010). The active ingredient in the Alamar Blue assay is resazurin (IUPAC name: 7-hydroxy-10-oxidophenoxazin-10-ium-3-one, also known as diazo-resorcinol, azoresorcin, resazoin, or resazurine); it enables accurate time course measurements, has high sensitivity and linearity, involves no cell lysis, is water-soluble, ideal for use with post-measurement functional assays, flexible (can be used with different cell models), scalable, can be used with fluorescence- and/or absorbance-based instrumentation platforms, and is non-toxic and non-radioactive, meaning it is safe for the user and the environment (O'Brien, Wilson, Orton, & Pognan, 2000; Rampersad, 2012).

## PURIFICATION AND ACTIVATION OF COMPOUNDS

Following bioassay screening, the crude extracts of cocoa plant parts with potential anti-cancer activity are further investigated to identify the chemical structure of the potential anti-cancer agents. The first step to purifying and characterizing the potential anti-cancer agents is fractionation and purification, a multi-step procedure. Fresh or dry samples are ground into powder, followed by (i) organic solvent, water, and $CO_2$ extraction, (ii) solvent partitioning to remove less polar and/or more polar compounds, (iii) fractionation (flash chromatography, counter-current chromatography, or low-pressure column chromatography [CC] on silica or alumina), and (iv) final purification by thin-layer chromatography (TLC) and/or high-performance liquid chromatography (HPLC) (Figure 4) for removing more and less polar compounds (Dinan, Harmatha, & Lafont, 2001). A plant crude extract contains a complicated mix of several bioactive compounds and potential anti-cancer agents. Therefore, it is often necessary to initially fractionate the crude extract into various discrete fractions containing a group of compounds with similar polarities or molecular sizes using CC, prep-HPLC, and solid-phase extraction (SPE). However, the initial fractionation should not generate too many fractions from any crude extract, as the target compound may be divided between these fractions, such that only low concentrations of potential anti-cancer agents are produced, which might evade detection or not exhibit detectable anti-cancer activity in bioassay-guided fractionation (Sarker & Nahar, 2012). The most important factor to be considered before designing an isolation protocol is the nature of the target compound present in the crude extracts or fractions. The general features of the molecule that can aid isolation process design include solubility (hydrophobicity or hydrophilicity), acid–base properties, charge, stability, and molecular size. It is easy to obtain information from the literature on the chromatographic behavior of the target compound if a known compound is isolated from the same or a new source, and the most appropriate method can be selected. However, for as yet unknown reasons, it is more difficult to design an isolation protocol for a crude extract. For discovering unknown anti-cancer agents, it is advisable to perform phytochemical testing for the presence of compounds such as phenolics, steroids, alkaloids, and flavonoids (Sarker, Latif, & Gray, 2005).

Subsequently, the isolated potential anti-cancer agents are identified or characterized by conclusive structure elucidation methods. However, elucidating the structure of natural products is generally time-consuming, and sometimes can be the bottleneck in anti-cancer research. With established anti-cancer agents, this may not pose significant difficulty, but it can certainly be challenging if the compounds are new entities. Many spectroscopic methods can be used to obtain valuable information of the chemical structures of compounds, but interpreting these spectra requires specialist spectroscopic knowledge, structure elucidation skills, sound understanding of natural products chemistry, and above all, a great deal of patience (Duarte et al., 2012). As mentioned previously (Salim, Chin, & Kinghorn, 2008; Sarker & Nahar, 2012), past drug discovery from plant bioactive compounds is time-consuming, and depending on the complexity of the structures, identifying the structures of active compounds from an extract could take weeks, months, or even years. Currently, bioassay-guided fractionation has become significantly faster due to improvements in instrumentation such as ultraviolet–visible spectroscopy (UV–vis), which provides information on chromophores present in the molecule; infrared spectroscopy (IR) provides information on different functional groups; mass spectrometry (MS) yields information on molecular mass, molecular formula, and fragmentation pattern. The most commonly used MS methods are electron impact MS (EIMS), chemical ionization MS (CIMS), electrospray ionization MS (ESIMS), fast atom bombardment MS (FABMS), and matrix-assisted laser desorption ionization (MALDI). Nuclear magnetic resonance (NMR) yields information on the number and type of protons and carbons (and other elements, such as nitrogen and fluorine) present in the molecule, and the relationships among these atoms are categorized using one- and two-dimensional NMR techniques.

Cocoa beans are a rich source of flavonoids, especially epicatechin, and catechin and its polymer forms, the monomer of which is procyanidin. Different plant varieties and parts yield different polyphenol content (Jayasekera, Molan, Garg, & Moughan, 2011). As plant extracts usually occur as a combination of bioactive compounds or phytochemicals with different polarities, their separation and purification remains a big challenge for bioactive compound identification and characterization. The common practices in bioactive compound purification and identification involve separation techniques such as TLC, CC, and HPLC, which are used to obtain pure compounds for identification (Martin & Guiochon, 2005; Sticher, 2008). The purification, identification, and structure elucidation methods for polyphenols in cocoa

components are the focus in the analytical methods that emphasize HPLC, the typical method of choice due to its high resolution, efficiency, and reproducibility and relatively short analysis time, which is not limited by sample volatility. Moreover, HPLC can be coupled to a variety of detectors such as UV–vis, photodiode array (PDA), fluorescence, electrochemical (ECD), and MS. However, it is worth considering TLC as a screening method and capillary electrophoresis (CE) as a promising tool (Wollgast & Anklam, 2000). As reported previously (Calderón, Wright, Hurst, & van Breemen, 2009), rapid methods are needed to evaluate and compare food products for their antioxidant benefits, and new assays based on liquid chromatography–MS (LC-MS) have been developed for identifying and quantitatively analyzing antioxidants in complex natural product samples such as food extracts. Assay analysis of cocoa powder methanolic extracts determined that procyanidins were the most potent antioxidant species. These species were identified using LC-MS, LC-MS-MS, accurate mass measurement, and comparison with reference standards. Catechin and epicatechin were the most abundant antioxidants, followed by their dimers and trimers. The new LC-MS assay facilitates the rapid identification and determination of the relative antioxidant activity of individual antioxidant species in complex comounds in cocoa. Gas chromatography–MS (GC-MS) also can be used to identify potential anti-cancer compounds from non-edible parts of *T. cacao* (Zainal, Abdah, Taufiq-Yap, & Rosmin, 2014). NMR identification of new or unknown compounds is also necessary, as reported previously (Alemanno, Ramos, Gargadenec, Andary, & Ferriere, 2003), where the three most abundant new polyphenolic compounds from cocoa, i.e., N-trans-caffeoyl-L-DOPA, N-trans-p-coumaroyl-L-tyrosine, and N-trans-caffeoyl-L-tyrosine, were isolated and characterized using $^{1}$H- and $^{13}$C-NMR.

## EVALUATION FOR APOPTOTIC EFFECTS OF COCOA ACTIVE COMPOUNDS

The purified potential anti-cancer compounds from *T. cacao* are evaluated based on their apoptotic effects. There are many articles on the induction of apoptosis in cancer cells by cocoa bean extract and other medicinal plant extracts (Martin et al., 2013; Maskarinec, 2009). Apoptosis, an active physiological process involved in the cellular self-destruction of unwanted cells, is disrupted in cancer cells and is

characterized by distinct morphologic changes, including cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation, and apoptotic body formation (Shafi et al., 2009). Therapeutic applications of apoptosis are considered a good model for evaluating potential anti-cancer agents from cocoa bean extract, and is a very important study for studying the apoptotic activity induced by anti-cancer agents. This review discusses the *in vitro* apoptotic activity of cocoa bean polyphenol compounds. As an example, polyphenols can affect the overall process of carcinogenesis through several mechanisms (D'Archivio et al., 2008b). In particular, polyphenols are involved in counteracting the occurrence of oxidative stress, contributing to the prevention of cancer onset and development. Furthermore, polyphenol modulation of oxidative stress in cancer cells affects signal transduction, activation of redox-sensitive TFs, and the expression of specific genes that influence cell proliferation and apoptosis (Figure 5). In addition, the evidence indicates that polyphenols can directly modulate different points of the apoptotic process and/or the expression of regulatory proteins, such as cytochrome c release with subsequent caspase-9 and caspase-3 activation, increased caspase-8 and truncated Bid (t-Bid) levels, Bcl-2 and Bcl-XL downregulation, enhanced Bax and Bak expression, and NF-κB modulation (Cho et al., 2008; D'Archivio et al., 2008a; Rodríguez-Ramiro, Ramos, Bravo, Goya, & Martín, 2011).

Brisson, 2008 also mentioned that cell death induced by the epigallocatechin-3-gallate (EGCG) group of polyphenolic compounds involves the stimulation of diverse protein kinases and modulation of the cell survival/cell death genes. Changes such as increased p53 and Bax, decreased Bcl-2 and Bcl-XL, and caspase-9 activation suggest that they interfere with the mitochondrial pathway, leading to apoptosis. These events suggest an effect on the mitochondrial pathway of apoptosis. In addition, a study of pentameric procyanidin from *T. cacao*, which inhibited breast cancer cells, showed that the mitochondrial effects caused by the pentamer trigger growth arrest or apoptotic or nonapoptotic cell death processes. Studies using the novel mitochondriotoxic small molecule F16 have suggested that compounds causing mitochondrial depolarization inhibit tumor growth of breast cancer cells through G1 arrest, apoptosis, or necrosis, depending on the genetic background of the cell (Ramljak et al., 2005). Typically, induction of apoptosis is determined using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Gunadharini, Elumalai, Arunkumar, Senthilkumar, & Arunakaran, 2011), cell cycle analysis (Marco Arlorio et al., 2009), DNA fragmentation (Machana, Weerapreeyakul, & Barusrux, 2012), western blotting

(Hsu et al., 2011), mitochondrial membrane potential assay (Ramljak et al., 2005), or caspase-3 activity assay (Alshatwi et al., 2011).

## CONCLUSION

Cocoa is a high–nutritional value food with bioactive compounds that have been demonstrated as effective in some physiological and pathophysiological processes, and previous studies have suggested the efficacy of cocoa as a potential antiproliferative agent. Screening new anti-cancer agents from cocoa plant parts, especially cocoa bean, depends on the quality of sample collection, storage, extraction, and purification. There is no specific technique for discovering the constituents of the complex mixture present in *T. cacao* plant part extracts. However, broad separation can be achieved by fractionation with organic solvents and chromatographic techniques. An appropriate protocol for extraction, bioassay-guided fractionation, and purification can be designed only when the targeted compound has been determined and more information has obtained on the chemical and physical properties of the active compounds for isolation. For unknown active compounds in the course of discovering anti-cancer agents, trial-and-error extraction and purification are sometimes necessary for identifying the best possible method of discovery. To reduce the cost, time, and solvents used, techniques based on green analytical chemistry should be used for extracting and purifying potential anti-cancer agents from *T. cacao*.

NMR, LC-MS and GC-MS structural characterization plays an important role in anti-cancer agent discovery, as more sensitive and faster structural analysis methods expedite such discoveries. These techniques also yield valuable preliminary information about the content and nature of potential anti-cancer compounds, which is very useful when there are numerous samples to be processed, avoiding unnecessary isolation of compounds. The MTT assay produces highly reproducible results in the assessment of cell culture viability and is a valid, inexpensive, rapid, and simple system that is potentially useful for *in vitro* assays of human cancer cell lines. Several studies have indicated that plant anti-cancer agents act either through interaction with the cell cycle or by activating an apoptotic pathway. As multiple mechanisms can initiate apoptosis, the targets of plant substances could be diverse and may be

dependent on the cellular context. Several reviews have indicated the diversity and number of putative molecular targets. This is important for characterizing the cellular events leading to apoptosis in order to define the strategic use of potential anti-cancer agents in cocoa for treating human cancers.

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**Table 1:** *T. cacao* phenolic compounds and the cell line(s) tested.

| Phenolic compound | Biological effect | Reference |
|---|---|---|
| Polymer procyanidins | Caco-2 (colon) | Erlejman, Jaggers, Fraga, & Oteiza, 2008 |
| Procyanidin B2 | Caco-2 (colon), HL-60 (leukemia) | Erlejman et al., 2008 and Sakano et al., 2005 |
| Epicatechin | Caco-2 (colon), SH-SY5Y (neuroblastoma), HepG2 (hepatoma), MCF-7 (breast) | Erlejman et al., 2008, Ramiro-Puig & Castell, 2009, Granado-Serrano et al., 2010 and Rodgers & Grant, 1998 |
| 3'-O-methyl epicatechin | FEK4 (skin fibroblasts) | Basu-Modak et al., 2003 |
| Catechin | HepG2 (hepatoma), Caco-2 (colon), Int-407 (intestine) | Erlejman et al., 2008 and Cheng, Wu, Ho, & Yen, 2013 |