

LITERATURE REVIEW

2.1 *Hibiscus sabdariffa* L.

2.1.1 Botanical Description

Hibiscus sabdariffa L. or locally known as asam kumbang, asam susur, and asam paya is belonging to the large family of Malvaceae (Osman *et al.*, 2011). It is also commonly known as roselle (English), l'Oiselle (French), Spanish (Jamaica), karkade (Arabic), and Krachiap daeng (Thailand) (Maganha *et al.*, 2010).

Botanical Classification:

Kingdom : Plantae (Plants)
Subkingdom : Tracheobionta (Vascular plants)
Superdivision : Spermatophyta (Seed plants)
Division : Magnoliophyta (Flowering plants)
Class : Magnoliopsida (Dicotyledons)
Subclass : Dilleniidae
Order : Malvales
Family : Mavaceae (Mallow family)
Genus : Hibiscus L. (Rosemallow)
Species : *Hibiscus sabdariffa* L

Genus Hibiscus which belongs to Malvaceae has more than 300 known species which are used as ornamental plants. It can grow up to 5-7 feet in height, with lobed leaves sometimes used for greens. The narrow leaves and stems are reddish green in color. The main edible part is the fleshy sepal, called a calyx, surrounding the seed boll in the flower. The size of the calyx varies with each variety, but ranges from ½ to 1 ½ inches in diameter (James, 1994). The origin of *H. sabdariffa* is not fully known, but it is to believe to be native from India to Malaysia, where it is commonly cultivated, and must have been carried at an early date to Africa. It has been widely distributed in the Tropics and Subtropics of both hemispheres, and in many areas of the West Indies and Central America has become naturalized. It was first introduced to West Indies and cultivated mainly as an ornamental plant. *H. sabdariffa* is relatively a new crop in Malaysia. It was introduced into Malaysia in early 1990s. Its commercial planting was first promoted by the Department of Agriculture in Terengganu in 1993 and has now spread to other states. Presently, the planted area is quite small approximately 150 ha (Osman *et al.*, 2011).

Many parts of *H. sabdariffa* including seeds, leaves, fruits and roots are used in various foods. Among them, the fleshy red calyces are the most popular. The fleshy calyces of *H. sabdariffa* have been used in various countries as food or a food ingredient such as jellies, syrups, beverages, puddings, cakes, and wines (Christian *et al.*, 2006). The red persistent calyx of its flowers is the major component which has a sour taste and is commonly used in the preparation of cold and hot beverages and as a food colorant (Maganha *et al.*, 2010).

2.1.2 Medicinal Value

H. sabdariffa is used in many folk medicines. It is claimed as a Thai traditional medicine for kidney stones and urinary bladder stones (Hirunpanich *et al.*, 2006). *H. sabdariffa* also is said to have diuretic effects, used effectively in folk medicines for treatment of inflammatory diseases (Dafallah & Al-Mustafa, 1996), and cancer (Chewonarin *et al.*, 1999). The positive effect of *H. sabdariffa* extract consumption to decrease blood pressure has been proved in study on both man and rats (Faraji *et al.*, 1999 and Onyenekwe *et al.*, 1999). More recently, the antihypertensive action of *H. sabdariffa* has been confirmed with experimental hypertension (Odigie *et al.*, 2003). In addition, studies on humans also proved the anti-inflammatory effect of *H. sabdariffa* consumption (Beltrán-Debón *et al.*, 2010; Herrera *et al.*, 2004).

H. sabdariffa extract is also reported used as an antibacterial, antifungal, diuretic, uricosuric, and mild laxative substance (Farnworth & Bunyapraphatsara, 1992). In addition, the components of *H. sabdariffa* extract exhibit anti-tumor characteristics, immune-modulating and anti-leukemic effects (Muller & Franz., 1992 and Tseng *et al.*, 2000). Oil extracted from seeds of *H. sabdariffa* has been shown to have an in vitro inhibitory effect on *Bacillus anthracis* and *Staphylococcus albus* (Gangrade *et al.*, 1979). An ethanol extract of the dried leaves of the plant also has been shown enable to reduce aflatoxin formation (El-Shayeb & Mabrook, 1984), and to have an in vitro inhibitory effect against some fungi that include *Aspergillus fumigatus*, *Rhizopus nigricans* and *Trichophyton mentagrophytes* (Guerin & Reveillere, 1984).

2.1.3 Antioxidant Activity and Anticholesterol Effects of *H. sabdariffa*

The calyxes' part of *H. sabdariffa* have repeatedly been studied and shown to have positive health effects especially as a source of antioxidants. The calyx providing antioxidants even higher levels than traditional sources such as raspberries and blueberries (Juliani *et al.*, 2009). Anthocyanins and protocatechuic acid are among chemical constituents in *H. sabdariffa* that shown to have strong antioxidant (Lee *et al.*, 2002) and antitumor effects (Chang *et al.*, 2005 and Lin *et al.*, 2005).

The previous study found that the extract of dried *H. sabdariffa* calyx was shown to be active protect rat hepatocytes from tert-butyl hydroperoxide-induced cytotoxicity and genotoxicity by different mechanisms (Liu *et al.*, 2002 and Tseng *et al.*, 1997). Works done by Lin *et al.*, (2003) also found that protocatechuic acid from *H. sabdariffa* calyx was demonstrated to inhibit lipopolysaccharide-induced rat hepatic damage. Hibiscus protocatechuic acid has also been shown to inhibit the carcinogenic action of various chemicals in different tissues of the rat, including diethylnitrosamine in the liver. It was proposed that one of the mechanisms of this protective effect was associated with the scavenging of free radicals by antioxidant compounds exist in *H. sabdariffa* calyx (Taneka *et al.*, 1993). Administering the dried calyx extracts of *H. sabdariffa* was found significantly decreased serum cholesterol, triglycerides and LDL levels. Calyx of *H. sabdariffa* possesses both antioxidant effects against LDL oxidation and hypolipidemic effects in vivo. However, its mechanisms of action remain to be elucidated (Hirunpanich *et al.*, 2006).

2.1.4 Phytochemical Constituents

The calyx of *H. sabdariffa* has been known to contain many chemical constituents such as delphinidin-3-glucoxyloside, also known as hibiscin, the major anthocyanin in *H. sabdariffa* calyx. Others flavonoids constituents that exist in *H. sabdariffa* calyx are includes anthocyanins as cyanidin-3-rutinoside, delphinidin, delphinidin-3-monoglucoside, cyanidin-3-monoglucoside, cyanidin-3-sambubioside, cyanidin-3,5-diglucoside; the flavonol glycosides hibiscetin-3-monoglucoside, gossypetin-3-glucoside, gossypetin-7-glucoside, gossypetin-8-glucoside and sabdaritrin. Alkaloids, ascorbic acid, b-carotene, anisaldehyde, arachidic acid, citric acid, malic acid, tartaric acid, glycinebetaine, trigonelline,; quercetin, protocatechuic acid, pectin, polysaccharides, mucopolysaccharides, stearic acid and wax also reported to be exist in *H. sabdariffa* calyx (Hirunpanich *et al.*, 2005).

The calyx yielded 65% (dry weight) of mucilage, which on hydrolysis gave galactose, galacturonic acid and rhamnose. These molecules are bioactive in several biological models and responsible by the pharmacological effects presented by the extracts of this species. Various antioxidant constituents are found in the calyx and flower petals of roselle, such as anthocyanins, quercetin, ascorbic acid, β -sitosteroid glycoside and protocatechuic acid (Tseng *et al.*, 1997). Furthermore, *H. sabdariffa* calyx contained polyphenolic acids (1.7% dry weight), flavonoids (1.43% dry weight) and anthocyanins (2.5% dry weight) (Tsuda *et al.*, 2000).

2.1.5 UKMR-1 and UKMR-2 variety

Conventional hybridization in *H. sabdariffa* is difficult due to its cleistogamous nature of reproduction. To overcome this limitation, an intensive mutation breeding program using different doses of gamma radiation was conducted (Osman *et al.*, 2011). A research program was initiated at UKM in cooperation with Malaysian Nuclear Agency in 1999 to increase genetic variation and germplasm accession for breeding programs. Through this research program, new variety of *H. sabdariffa* namely UKMR-1 and UKMR-2 were produced.

Morpho-agronomic analysis of UKMR-1 and UKMR-2 varieties done by Osman *et al.*, (2011) found that these new variety showed better performance than Arab variety for forth parameters of plant characteristics which are number of branches per plant, number of fruits per branch, number of fruits per plant and weight of capsule per plant, whereas in plant height and canopy diameter they showed lower performance than Arab variety.



Figure 2.1: Arab variety



Figure 2.2: UKMR-1 variety



Figure 2.3: UKMR-2 Variety

2.2 Atherosclerosis

2.2.1 Development of Atherosclerosis

Hardening of the arteries wall or also called atherosclerosis is a condition when fat, cholesterol, and other substances accumulate in the walls of arteries and form hard structures called plaques. Over the time, these plaques can block the arteries and cause problems throughout the body. Atherosclerosis is assumed one of the major causes for cardiovascular mortality. Plasma lipoprotein profiles in blood serum play an important role in the development of atherosclerosis. Proper maintenance of the high density lipoprotein (HDL) level and reduction of the low density lipoprotein (LDL) level has been a major goal in treating cardiovascular diseases (Xia *et al.*, 1996).

LDL and HDL have opposite roles in body cholesterol regulation. LDL is always labeled as ‘bad’ lipoprotein due to its relationship in development of atherosclerosis. There is general agreement that the presence of small dense LDL is associated with an increased risk for coronary heart disease. Small dense LDL may play a role in the development of coronary heart disease due to its physiological properties, e.g. its high susceptibility to oxidation (Graaf *et al.*, 1991 and Chait *et al.*, 1993). Oxidation of low-density lipoprotein (LDL) is believed to be an important step in the atherogenic process. Lots of research on atherosclerosis reported elevated levels of oxidized LDL (Ox-LDL) in plasma of patients with atherosclerosis cardiovascular disease (Wang *et al.*, 2007). Other risk factors which contribute to coronary heart disease (CHD) are age, blood pressure, cigarette smoking, diabetes, lack of physical activity, obesity and atherogenic diets (Anderson *et al.*, 1991).

As oppose to LDL, HDL is called ‘good’ lipoprotein. A number of reports have indicated that raising high density lipoprotein (HDL), can result in significant cardiovascular benefit (Sirtori and Fumagalli, 2006). The protective effect of HDL toward atherosclerosis has been attributed primarily to its role in reverse cholesterol transport, the process whereby cholesterol is brought from peripheral cells to the liver for excretion in the bile. Recently, attention is being given to the possible anti-inflammatory activity of HDL, which may be mediated in part by enzymes and apolipoproteins that can inactivate LDL-derived oxidized phospholipids or prevent their formation (Navab *et al.*, 2001).

There are increasing evidence to suggest an association between inflammation and the risk for cardiovascular disease. Pathologic studies have found that atherosclerotic lesions contain infiltrates associated with inflammation (Van Lenten *et al.*, 2001). The complex anti-inflammatory effect of HDL is related in part to the activity of HDL-associated enzymes. These include paraoxonase, which can prevent the oxidative modification of LDL (Reddy *et al.*, 2001) and platelet-activating factor (PAF) acetylhydrolase, which hydrolyzes PAF, a proinflammatory phospholipids produced by activated platelets, leukocytes, and endothelial cells. Levels of both enzymes have been shown to decrease during the acute-phase response in mice (Tjoelker *et al.*, 1995)

The positive effect of antioxidant in preventing the formation of atherosclerosis is widely studied. Antioxidant substances are believed enable to suppress the onset and development of atherosclerosis. Compounds such as probucol, flavonoids and phenolic compounds have also been shown to have antioxidative effects and effective in preventing the formation and progression of atherosclerosis (Anderson *et al.*, 1995; Diaz *et al.*, 1997 and Chrysselis, 2000). These antioxidants were proved to give positive effect by prevent

LDL from oxidative damage in vitro and eventually interrupt the progression of atherosclerosis (Jialal *et al.*, 1990 and Sullivan *et al.*, 1990).

Besides antioxidant, 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors (Statins) are drug used to prevent atherosclerosis development. Statins are one class of many drugs used to lower the level of cholesterol in the blood by reducing the biosynthesis of cholesterol by the liver. Statins block the HMG-CoA reductase enzyme in the liver that is responsible for making cholesterol (Langsjoen, 2004). HMG-CoA reductase is membrane-bound enzyme found in the liver. It is involve in reduction process of HMG-CoA to mevalonate, the precursor in biosynthesis of cholesterol (Brown and Goldstein, 1980). HMG-CoA reductase is considered to be the rate-limiting enzyme of cholesterol synthesis and the pharmacological target of all statins (Ridker, 2003). By blocking the HMG-CoA reductase enzyme, the level of cholesterol in blood serum can be reduced and eventually lowering the risk of atherosclerosis development. There are many types of HMG-CoA reductase inhibitors drug available in the market such as Lovastatin, Pravastatin, Simvastatin, Fluvastatin, Atorvastatin and Rosuvastatin.

2.2.2 High Density Lipoprotein (HDL) and Low Density Lipoprotein (LDL)

The transportation of cholesterol, triglycerides and other water insoluble lipids in the blood circulation is achieved by packing them into water-soluble, mostly spherical particles that are called lipoproteins. Plasma lipoproteins are spherical particles of complexes of various lipids and specific proteins. The outer surfaces of these particles are made up of phospholipids, free cholesterol and protein. The inner core containing mostly esterified cholesterol and triglyceride. These plasma lipoproteins have traditionally been grouped into four major classes: the chylomicrons, the very low density lipoproteins (VLDL), the low density lipoproteins (LDL), and the high density lipoproteins (HDL). Each lipoprotein has a distinct composition of lipids and specific structural proteins that are called apolipoproteins (Timmins *et al.*, 2005).

The primary function of HDL is to transport cholesterol from peripheral tissues to the liver where it is metabolized. This process known as reverse cholesterol transport, has been proposed to be a cardiovascular protective mechanism. The determination of serum HDL cholesterol level is a useful tool in identifying at-risk patients. This is patients with low levels of HDL cholesterol are generally considered to be at increased risk for coronary artery disease (Vaisar *et al.*, 2007).

Nearly 80% of the plasma HDL is synthesized mainly by the liver and to a lesser extent ~20% by the intestine (Singaraja *et al.*, 2006 and Timmins *et al.*, 2005). HDL is consists of spherical particles ranging from about 5 to 15 nm in diameter. The composition of HDL is made up of approximately 50% apolipoprotein and 50% lipid moieties. The interior of HDL contains water insoluble lipids consisting of cholesteryl esters (CE) and a small amount of triglycerides (TG). On the surface of HDL, relatively polar or water

soluble lipids like phospholipids, a portion of the free cholesterol and apolipoproteins are found. The major protein component of HDL is apolipoprotein A-I (apoA-I) followed by apoA-II, whereas other proteins are minor constituents. As many as 60 other non-structural proteins have been reported to be associated with HDL particles but the roles of these proteins in concert with HDL are currently not known (Vaisar *et al.*, 2007).

The main function of LDL is the carriers of cholesterol to the adrenals and adipose tissues. The cholesterol carried in LDL particles is known as LDL cholesterol. The composition of LDL is made up of about 25 % protein and 45 % cholesterol (Pullinger *et al.*, 2002). Once the LDL cholesterol reaches the adrenals and adipose tissue surface, there are receptors requiring apo B100 that are able to take in the LDL by a similar process to that occurring in liver. Within these tissues, the cholesterol esters are hydrolyzed to yield free cholesterol, which is incorporated into the plasma membranes as required. Any excess cholesterol is re-esterified by an acyl-CoA cholesterol acyltransferase for intracellular storage (Russell, 2009).

The mechanisms that manage and utilize LDL are tightly controlled systems evolved to distribute cholesterol through the circulatory system and into cells that require extracellular cholesterol to repair cell membranes. Unfortunately, LDL-cholesterol does not always reach its most appropriate destination, but rather accumulates in artery walls and causing atherosclerosis, the leading cause of death and disability in the developed world (Yusuf *et al.*, 2001). For this reason, LDL cholesterol is always called as bad cholesterol. The quantity of circulating LDL in blood serum is a well-known risk factor for heart disease, and is the primary focus of most lipid lowering therapies (Koivisto *et al.*, 1993). The pathogenicity of LDL and likelihood of atherosclerotic development are heavily influenced by genetic composition of gene products involved with LDL metabolism.

Patients with genetic defects that cause severely elevated LDL have familial hypercholesterolemia, which affects approximately 1:500 people, and is the consequence of mutations in the low density lipoprotein receptor (LDLR), ApoB, and other genes. In normal individuals, approximately 50% of LDL variation is genetic (Goodwin *et al.*, 2000).

2.2.3 Cholesterol

Cholesterol is a chemical compound that is naturally produced by the liver in the body and is a combination of lipid (fat) and steroid. It is an essential building block for structural component of cellular membranes and is converted into steroid hormones and bile acids, which fulfill important roles in the mediation of cellular signals, the control of gene expression, the absorption of fat from the small intestine and the secretion of liver waste products. Cholesterol is also main structural component for hormones like estrogen and testosterone (Kleemann & Kooistra, 2005; Chiang, 2003 and McKenna & O'Malley, 2002).

About 80% of the body's cholesterol in mammalian is produced by the liver, while the rest comes from diet (Maxfield and Wustner, 2002 and Kleemann & Kooistra, 2005). Dietary cholesterol comes mainly from poultry, fish, and dairy products. Organ meats, such as liver, are especially high in cholesterol content, while foods of plant origin contain no cholesterol. After digested, dietary cholesterol is absorbed from the intestine and stored in the liver. The liver is able to regulate cholesterol levels in the blood stream and can secrete cholesterol if it is needed by the body. In the intestine, dietary cholesterol and triglycerides are packaged into chylomicrons which enter the circulation via the lymph. The capillary vessel wall of peripheral tissues contains lipoprotein lipase, which hydrolyzes triglycerides in the core of chylomicrons into free fatty acids. The resulting chylomicron remnant particles are relatively enriched in cholesterol and are rapidly cleared by the liver via apolipoprotein E-mediated binding to specific cell surface receptors, i.e. low density lipoprotein receptor (LDLR) and low density lipoprotein receptor-related protein (LRP) (Kleemann & Kooistra, 2005).

Most of the cholesterol needed in the body synthesized by liver. The synthesis of cholesterol is begin in the cytoplasmic compartment, thiolase combines two acetyl-CoA units to acetoacetyl-CoA which is further combined by HMG-CoA synthase with a third acetyl-CoA unit to form the six carbon (C6) intermediate HMG-CoA. HMG-CoA is transported to the endoplasmic reticulum membrane where it is reduced to mevalonate by the membrane-bound enzyme HMG-CoA reductase using NADPH as reductant (Brown & Goldstein, 1980)

Mevalonate is subsequently decarboxylated to form C5 intermediate, isoprene, which is also involved in the synthesis of ubiquinone, vitamine K and carotenoids. Six isoprene units are used to generate the C30 compound squalene. During squalene synthesis, the intermediate farnesyl-pyrophosphate (F-PP) and the side product geranylgeranyl-pyrophosphate (GG-PP) are formed. GG-PP and F-PP are required for the posttranslational modification of proteins and serve as lipid attachments. In the last stage of cholesterol synthesis, squalene is reduced in the presence of molecular oxygen and NADPH to squalene epoxide and cyclized to form lanosterol. Lanosterol is finally reduced and demethylated to form the end product, cholesterol (Haslinger *et al.*, 2003)

2.2.4 Triglycerides

A molecule of triglyceride is composed of a backbone of the alcohol glycerol to which three fatty acids (tri) are bound. Any combination of saturated, monounsaturated, or polyunsaturated fatty acids can be in a triglyceride molecule (Mierzejewski, 2004). Triglycerides are the chemical form in which most fat exists in food as well as in the body. They're also present in blood plasma and, in association with cholesterol, form the plasma lipids. Triglycerides in plasma are derived from fats eaten in foods or made in the body from other energy sources like carbohydrates. Calories ingested in a meal and not used immediately by tissues are converted to triglycerides and transported to fat cells to be stored. Hormones regulate the release of triglycerides from fat tissue so they meet the body's needs for energy between meals (Welson, 2006).

Excess triglycerides in plasma are called hypertriglyceridemia. It's linked to the occurrence of coronary artery disease in some people. Elevated triglycerides may be a consequence of other disease, such as untreated diabetes mellitus. Like cholesterol, increases in triglyceride levels can be detected by plasma measurements. These measurements should be made after an overnight food and alcohol fast (Miller *et al.*, 1998). The status of high plasma triglyceride concentrations as risk markers for the presence of coronary heart disease (CHD) (Stampfer *et al.*, 1996), and ischemic stroke (Tanne *et al.*, 2001) has been examined in a number of studies. Hypertriglyceridemia is often associated with low high-density lipoprotein (HDL) cholesterol that contributes to cardiovascular disease and stroke (Bloomfield *et al.*, 2001). Hypertriglyceridemia may be associated with rheological and impaired fibrinolytic mechanisms that can contribute to the development of atherothrombosis (Humphries *et al.*, 1994). Plasma triglycerides are carried in triglyceride-rich lipoproteins which not only promote atherogenesis but complicate thrombotic events as

well. Chylomicron-derived and large VLDL particles, apart from accumulating in arteries, can be readily taken up by macrophages leading to foam cell formation and may also lead to atherosclerotic disease through effects on blood coagulation and fibrinolysis (Altan *et al.*, 2006)

2.3 Antioxidant

Antioxidant is a substance that slows or inhibits oxidation reaction, especially in biological materials or within cells, thereby reducing spoilage or preventing damage. The main function of antioxidant is trapping the free radical particularly reactive oxygen species (ROS) and reactive nitrogen species (RNS) which involved in the pathogenesis of several chronic and degenerative diseases such as cardiovascular diseases, inflammation, neurodegenerative diseases, aging-related disorders and cancer. Antioxidant works by slowing or preventing the oxidation of other molecules by free radical. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions produce free radicals, which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves (Droge, 2002).

In general, free radicals are part of life as human consume some 3.5 kg of oxygen each day, some of which is not completely reduced, thus leading to the formation of free radicals and other reactive oxygen species (ROS) such as super-oxide, hydroxyl radical, peroxy radical, and alkoxy radical, as well as hydrogen peroxide and other peroxides. Therefore, several kilograms of peroxides and other ROS may be produced in the body each year. As mentioned earlier, free radical is responsible for several chronic and degenerative diseases because the highly reactive free radical can readily react with various biological macromolecules such as lipids, proteins, and DNA which eventually cause mutation, peroxidation of membrane lipids, and protein destruction. Reactive oxygen species and reactive nitrogen species are secondary messengers in normal physiological functions of the organism and also participate in various regulatory redox-mechanisms but

an overproduction of these species can overwhelm protective enzymes and cause destructive and lethal cellular effects (Halliwell, 1996).

In healthy individuals, free radicals and other ROS are neutralized by antioxidant defense mechanisms in the body, including superoxide dismutase and glutathione. However, endogenous systems may not provide sufficient protections in individuals suffering from certain diseases; in such cases help from dietary sources is important (Lee *et al.*, 2004). To overcome this problem, antioxidant compound in food has been promoted as an important role as a health-protecting factor. Hence in recent years, lots of researches have been conducted in order to investigate the natural antioxidant activity of plant extracts. Phenolic compounds are among phytochemicals in plant extracts that may render their effects via inhibit the oxidation reaction caused by oxidative stress and relief its consequences. (Shahidi, 2000).

The antioxidative effect of phenolics compound in foods is due to a direct free radical scavenging activity, reducing activity and an indirect effect arising from chelation of prooxidant metal ions. The chelation of metal ions generally requires ortho-dihydroxylation on the phenyl ring in phenolic acids and flavonoids or the presence of a 3- or 5-hydroxyl group in flavonoids (Halliwell, 1996). Among plant materials, fruits and vegetables contain phenolics, mainly belonging to the flavonoids family, but also phenolic acids. Meanwhile, cereals contain a wide range of phenolic acids, belonging mainly to the benzoic acid and cinnamic acid groups. Phenolic acids are different from other phenolic compounds by bearing acidic properties due to the presence of a carboxylic acid group. Ferulic acid and p-coumaric acid are the major phenolic acids found in many cereals, including barley (Liyana-pathirana & Shahidi 2004). Phenolic compounds in cereal grains can be found in the free, soluble conjugate or esterified and insoluble-bound forms. It is

reported that 74 and 69% of total phenolics present in rice and corn, respectively, are in the insoluble-bound form (Adom & Liu, 2002).