

1. INTRODUCTION

1.1 INTRODUCTION

Roselle (*Hibiscus sabdariffa* L.) belongs to the family Malvaceae, locally called “karkade”, and is an important annual crop grown successfully in tropical and sub-tropical climates (Copley, L.S. (1975)). The commercially important part of the plant is the fleshy calyx (sepals) surrounding the fruit (capsules). The whole plant can be used as beverage, or the dried calyces can be soaked in water to prepare a colorful cold drink, or may be boiled in water and taken as a hot drink. It also has some medicinal properties (National Biodiversity Action Plan (N.B.A.P.) (1999)). The seeds contain 17.8–21% non-edible oil (Ahmed, A.K. (1980)) and 20% protein, and are sometimes used for animal feed (Ahmed, A.H.R and Nour, A.M. (1981)).

Roselle is a flexible plant with a number of uses. It is intercropped with crop staples such as sorghum and sesame, or planted along field margins. It requires little care. Its leaves, seeds, capsules and stems are used in traditional medicines. In rural areas women are usually responsible for growing roselle. They add value to the crop by developing products for market (McClintock, N. (2004) Mclean, K. (1973) and Wilson, F.D. and Menzel, M.Y. (1964) reported that *Hibiscus Sabdariffa* is a tetraploid ($2n = 4x = 72$), whose chromosomes are related to the diploid ($2n = 2x = 36$) *Hibiscus cannabinus*. The two botanical types of roselle are *Hibiscus Sabdariffa* var. *sabdariffa*, grown for its fleshy, shiny-red calyx, and *Hibiscus Sabdariffa* var. *altissima* (Purseglove, J.W. (1974)) grown for its phloem fiber. Despite its potential economic importance, karkade has received little attention and there is a lack of information regarding its genetics, breeding and production, particularly under rain-fed condition.

Flavonoids and polyphenols are heterocyclic molecules that have been associated with beneficial effects on human health, such as reducing the risk of various diseases like cancer, diabetes, and cardiovascular and brain diseases.

Protocatechuic acid (PCA) is a type of widely distributed naturally occurring phenolic acid. PCA has structural similarity with gallic acid, caffeic acid, *vanillic* acid, and syringic acid which are well-known antioxidant compounds. More than 500 plants contain PCA as active constituents imparting various pharmacological activity and these effects are due to their antioxidant activities, along with other possible mechanisms, such as anti-inflammatory properties and interaction with several enzymes. Over the past two decades, there have been an increasing number of publications on polyphenols and flavonoids, which demonstrate the importance of understanding the chemistry behind the antioxidant activities of both natural and synthesized compounds, considering the benefits from their dietary ingestion as well as pharmacological use. This work aims to review the pharmacological effects of PCA molecules in humans and the structural aspects that contribute to these effects.

1.2. Objectives

- To isolate protocatechuic acid from *H. sabdariffa* calyces in pure form.
- To investigate its antimicrobial activities.

2. Literature Review

2.1. *Hibiscus sabdariffa*

2.1.1. Morphological Characters of *H. Sabdariffa*

Hibiscus sabdariffa is a member of the Malvaceae family and is native to tropical Africa. It is an annual herb that grows up to 1.8 meters or more. The stems are glabrous; the leaves ovate with the upper leaves being 3 to 5 palmately lobed. The flowers are axillary or in terminal racemes, the petals are white with a reddish center at the base of the staminal column. The calyx enlarges at maturity and the fruit is fleshy and bright red (Mc Caleb, R., (1996)); Hibiscus is relatively hardy and grows well in most soils that are well drained. It requires 4 to 8 months with a nighttime temperature higher than 21°C. In addition it requires 13 hours of sunlight during the first 4 to 5 months of growth to prevent premature flowering. Hibiscus requires a monthly rainfall ranging from 130 to 260 mm in the first 3 to 4 months of growth. Dry periods can be withstood in the last months of growth. Rain and high humidity during harvest and drying can downgrade the quality of the calyces and reduce the yield (Morton, J.F., (1987)).



Fig. 2.1. Photo of Hibiscus Sabdariffa calyx

2.1.2. Origin

Roselle may have been domesticated in western Sudan before 4000 BC (Wilson, F.D. and Menzel, M.Y. (1964)). It was first recorded in Europe in AD 1576. It seems to have been carried from Africa to the New World by slaves for use as a food plant. Roselle was called Jamaican sorrel in 1707 in Jamaica, where the regular use of the calyxes as food seems to have been first practiced (Wilson, F.D. and Menzel, M.Y. (1964)). The use of the plant as “greens” was known in Java as early as 1658 ((Lainbourne, J. (1913)). Taken to the New World, roselle was cultivated in Mexico, parts of Central America, the West Indies, and in southern Florida, Texas and California in the late 19th century. It is now grown for culinary purposes in much of the tropical world. The use of *H. sabdariffa* for fiber seems to have developed in regions other than Africa (Wilson, F.D. and Menzel, M.Y. (1964)). Most breeding of roselle has been for its fiber yield (Duke, J.A. (1993)).

Sudan is presently the major producer of roselle; however, farmers regard it as a famine food. When drought is expected, farmers prefer to cultivate roselle rather than cereals because of its hardiness under adverse conditions (Mohamad, *et al.*, (2002)). Roselle is grown for its calyces, which are exported from the Sudan, China and Thailand, and it is also grown for its calyces in Mexico. In the Sudan it is collected by goat-herding nomadic tribes, but the product is frequently inferior because of poor processing conditions. Nevertheless, the Sudanese product is attractively bright red, very acidic, and it is extremely popular in Germany, which imports most of the crop. Export prices for the 1992–93 season for Sudanese, Chinese and Thai roselle was of the order of \$US1700.00/t (Duke, and duCellier, J.L. (1993))

Karkade is grown in various parts of the Sudan, particularly Kordofan and Darfur. It is one of the cash crops cultivated by traditional farmers in Kordofan and Darfur States under rain-fed conditions, where large quantities are produced both for local consumption and for export. The total area under cultivation was estimated at 290,000 feddans (approximately 121,800 ha) in the 2000/2001 season, compared with 22,300 to 78,444 feddans (approx. 9370–32,950 ha) in the 1970s and 47998 to 59882 feddans (approx. 20,160–25,160 ha) in the 19'80s. The increased area raised production from 454 tons in the 1960s to 26,000 tons in the 1999/2000 season (El-Awad, H.O. (2001)).

Roselle is an important cash crop and a source of income for small farmers in western Sudan, especially in North Kordofan State. The crop is grown mainly by traditional farming methods, exclusively under rain-fed conditions (El Naimet *al.*, (2010)).

China and Thailand are also major producers, and control much of the world's supply. Thailand has invested heavily in roselle production and their product is of superior quality, whereas China's product, with less stringent quality control practices, is less reliable and reputable. The world's best roselle comes from the Sudan, but the quantity is low, and poor processing hampers quality. Mexico, Egypt, Senegal, Tanzania, Mali and Jamaica are also important suppliers but production is mostly used domestically (Mohamad, O., *et al.*,(2002)).

2.1.3. Uses

A Research Review on the Use of *Hibiscus Sabdariffa*

In 2007, a clinical trial showed that Hibiscus reduced cholesterol by 8.3% to 14.4% after just one month (Lin T *et al.*,(2007)). A total of 42 subjects were randomized to 3 groups for the study, conducted in Taiwan. The hibiscus extract capsules contained 500 mg of dried herb by macerating 150 g of hibiscus flowers in 6 L of hot water for 2 hours and then drying and filtering the extract. Group 1 received 1 capsule of extract 3 times daily (1,500 mg/day), group 2 received 2 capsules 3 times daily (3,000 mg/day), and group 3 received 3 capsules 3 times daily (4,500 mg/day) Overall, subjects in group 2 responded best to the hibiscus extract treatment. Groups 1 and 2, but not group 3, experienced a significant reduction in serum cholesterol levels at week 4, compared with baseline levels. In addition, group 2 experienced a significant reduction in serum cholesterol levels at week 2, compared with baseline levels. At week 2, there was a 42.9% responder rate in groups 1 and 3 and a 64.3% responder rate in group 2. By week 4, group 2 had a cholesterol reduction response from 71.4% of the subjects. In group 1, 50.0% were responders, and 42.9% subjects in group 3 were responders at week 4. It

appeared that group 2, taking 1,000 mg three times daily, was the optimum dose in achieving cholesterol reduction effects. While this study is small with a small number of subjects in each of the study groups, as well as a short duration of 4 weeks, there was indeed a clear effect with significant reductions in serum cholesterol seen as early as week 2, in the 1,000 mg tid group. Oddly enough, the responders in group 3, receiving the highest dose (4,500 mg/day), had the smallest response to the hibiscus extract with an average of 8.3% reduction at week 4. Group 1 received a 14.4% reduction at week 4.

In 2009, 60 Type 2 diabetics, mostly women, were given either Hibiscus tea from Saudi Arabia or black tea, 1 cup twice per day (Mozaffari-Khosravi H *et al.*, (2009)) Seven individuals withdrew from the study and after one month, mean HDL cholesterol increased significantly (48.2 mg/dl to 56.1 mg/dl) whereas a polipoprotein A1 and lipoprotein (a) were not significant. There was also a significant decrease in the mean of total cholesterol (236.2 to 218.6), LDL cholesterol (137.5 to 128.5), triglycerides (246.1 to 209.2) and Apo-B100 (80.0 to 77.3) in the Hibiscus group. Only HDLc showed a significant change in the black tea group (46.2 to 52.01). Something as simple as Hibiscus tea in diabetic is a welcomed intervention. Achieving a 7.6% decrease in total cholesterol, an 8.0% decrease in LDLc, a 14.9% decrease in triglycerides, a 3.4% decrease in Apo-B100, a 4.2% increase in Apo-A1 and a 16.7% increase in HDLc is no small accomplishment with merely two cups of tea per day.

Hibiscus extract was also studied in 222 patients, some with and some without metabolic syndrome (MS) (Gurrola-Diaz C *et al.*, (2010)). A total daily dose of 100 mg Hibiscus sabdariffa extract powder (HSEP) was given for one month to men and women, 150 without MS and 72

with MS. They were randomly assigned to a preventive diet, HSEP treatment or diet combined with HSEP treatment. The MS patients receiving HSEP had significantly reduced glucose, total cholesterol and LDLc and increased HDLc. A triglyceride lowering effect was seen in all groups but was only significant in the control group that was treated with diet. The triglyceride/HDLc ratio was also significantly reduced with HSEP in the control and MS groups, indicating an improvement in insulin resistance. It has been hypothesized that the anthocyanins regulate adipocyte function, which has definite and important implications for both preventing and treating metabolic syndrome. Due to both its hypolipidemic and hypotensive effects, Hibiscus extract would be an excellent option for individuals with metabolic syndrome.

A double-blind, placebo control, randomized trial in 69 subjects with elevated LDL and no history of coronary heart disease did not appear to show a blood lipid lowering effect from Hibiscus extract (Kuriyan R *et al.*,(2010)).

The treatment group received 1,000mg/day Hibiscus extract for 90 days in addition to dietary and physical activity. Body weight, serum LDL cholesterol and triglyceride levels decreased in both the extract and placebo groups, with no significant differences between the two. It is likely that the positive effects were due to dietary and exercise activity. One wonders why the results of this study were negative and the 3 previous studies above, showed positive results. The doses and product used in all four studies were different. One a tea, another used dried powdered flowers, another used a standardized extract powder of the sepals of the flowers, and this one, an ethyl alcohol/water extract, dried and then powder of the leaves. It is reasonable to consider that these different preparations would yield different results. With more consistent

product selection and dosages used in larger randomized trials, we would hope that this would clarify the best intervention to use.

Hypertension is another area indicated for the use of Hibiscus. The blood pressure lowering effects of sour tea (ST) =Hibiscus sabdariffa was compared with black tea (BT) in type II diabetics with mild high blood pressure (Mozaffari-Khosravi H *et al.*, (2009)).

Patients were randomly assigned to drink one cup of Hibiscus or black tea two times per day for one month. The average systolic blood pressure (SBP) in the hibiscus group decreased from 134.4 ± 11.8 mm Hg at the start of the study to 112.7 ± 5.7 mm Hg after 1 month. The average SBP changed from 118.6 ± 14.9 to 127.3 ± 8.7 mm Hg in the black tea group during the same time period. There were no statistically significant effects on the mean diastolic blood pressure in either group. This drop in systolic blood pressure is clinically relevant, decreasing systolic blood pressure in pre-hypertensive ranges, to normal systolic blood pressure. We do not know if systolic blood pressure would be lowered by one cup of Hibiscus tea in those with stage I or stage II hypertension.

A randomized, controlled, double-blind clinical comparison study was done of Hibiscus sabdariffa extract with lisinopril on patients with stage I or II hypertension (Herrera-Arellano A *et al.*,(2007)). Adried powdered Hibiscus extract was delivered in 250 mg of water containing a total of 250 mg anthocyanins from Hibiscus extract for 4 weeks and the lisinopril group received 10 mg/day. Results showed that the Hibiscus extract decreased blood pressure from 146/98 mm/Hg to 130/86 mm/Hg. Blood pressure reductions were lower than with lisinopril, but the Hibiscus extract did not modify plasma potassium levels and did not have the mineralocorticoid effects. Based on the study data, the authors concluded that the Hibiscus extract did have a significant

antihypertensive action, and through at least two mechanisms of action: diuretic effects likely as an aldosterone antagonist and ACE inhibitory effects. It was also reassuring to note in the study that the diuretic activity did not alter plasma potassium levels and did not have mineralocorticoid effects.

A Cochrane review of Hibiscus effects on blood pressure published in 2010 resulted in five articles (Ngamjarus *c et al.*,(2010)). The reviewers included randomized controlled trials (RCTs) of 3-12 weeks in duration that compared Hibiscus to either placebo or no intervention at all. All five of these studies found significant reductions in systolic blood pressure. While they could not draw reliable conclusions about the benefit of Hibiscus for controlling or lowering blood pressure in hypertensive patients, in the articles that met their inclusion criteria, they did state that beneficial effects were found in the treatment of hypertension with Hibiscus, but that well-designed, placebo-controlled RCTs were needed.

2.1.4. Clinical study and pharmacological activity of *H. sabdariffa*

2.1.4.1. Effect on blood pressure :

Intravenous injection of aqueous extracts of *H. sabdariffa* calyx to anaesthetized cats (Chen S-H, et al (1998)) and anaesthetized rats (Salama RB and Ibrahim SA. (1979) lowered blood pressure in a dose-dependent manner. This effect was resistant to a number of standard receptor blocking agents, but the hypotensive effect was partially blocked by (Chen S-H, et al (1998)), and atropine and antihistamine (H1 blockers) Therefore, the hypotensive action may be mediated, at least partially, by a cholinergic and/or histaminergic mechanism. Sectioning of the left and right vagi nerves did not have a significant effect on the fall in mean arterial blood It was also postulated that the hypotensive action of *H.*

sabdariffa could be ascribed to a direct vaso-relaxant effect (Salama RB and Ibrahim SA. (1979)). Another possible mechanism for the hypotensive activity may be inhibition of angiotensin I converting enzyme (ACE). The latter action has been demonstrated in vitro with a crude hydroethanol extract of *H. sabdariffa* calyces, and was ascribed to flavones present in the extract. In addition, a beneficial cardioprotective effect of this extract was shown in vivo, and was attributed to Flavonoids and anthocyanins (Ali MS et al.,(1991)). More recently, the antihypertensive action of *H. sabdariffa* has been confirmed in rats with experimental hypertension (Adegunloye BJ et al.,(1996)) and in spontaneously hypertensive rats (Jonadet M et al., (1990)) given the aqueous extracts at doses of 250–1000 mg/kg for up to 14 weeks. In a single clinical trial involving 54 patients with moderate essential hypertension, it was reported that daily consumption of an aqueous *H. sabdariffa* extract (two spoonfuls of blended ‘sour tea’ boiled in one glass of water for 20–30 min) resulted in about an 11% decrease in systolic and diastolic blood pressure 12 days after beginning the treatment. Three days after cessation of the treatment, the blood pressure rose again by about 6–8%. The authors did not investigate the possible mechanism(s) of action of the plant extract, but a diuretic, vasodilator and/or an inhibitory effect on ACE was postulated (Odigie IP et al., 2003)). The effectiveness of an aqueous extract of *H. sabdariffa* on mild to moderate hypertension was recently confirmed in a clinical trial involving 39 Mexican patients (Onyenekwe PC et al., (1999)).

The extract was made from 10 g dried calyx in 0.5 L water (9.6 mg anthocyanin content) and was given daily for 4 weeks before breakfast. For comparison, 36 hypertensive patients were given the ACE inhibitor, captopril (25 mg twice daily for 4 weeks). The extract treatment reduced

the systolic blood pressure from 139 to 124 mm mercury, and the diastolic from 91 to 80 mm mercury. These results were not significantly different from those obtained by captopril treatment. No adverse effects were found with either treatment, confirming the effectiveness and safety of the extract

2.1.4.2. Antioxidant and anticancer activity :

An 80% ethanol extract of *H. sabdariffa* was effective in reducing about 60%–90% of the mutagenicity induced by heterocyclic amines at a concentration of 12.5 mg/plate in the salmonella mutation assay. Below this dose, neither significant antimutagenic nor antibacterial effects were observed (Haji-Faraji M et al., (1999)). The extract of the plant also inhibited the formation of colon cancer at the initiation stage. Fractions of the ethanol extract of dried flowers of *H. sabdariffa* were evaluated by their capacity to quench 1, 1-diphenyl-2-picrylhydrazyl free radical and inhibiting xanthine oxidase activity. The ethyl acetate fraction of the ethanol extract showed the greatest ability of scavenging free radical and the chloroform fraction showed the strongest inhibitory effect on xanthine oxidase activity. The antioxidant activities of the various extracts were also investigated using a model of tert-butyl hydroperoxide-induced oxidative damage in rat primary hepatocytes. Both fractions were shown to be active, indicating that the extract of dried *H. sabdariffa* flowers protect rat hepatocytes from tert-butyl hydroperoxide-induced cytotoxicity and genotoxicity by different mechanisms (Herrera-Arellano A et al., (2004))

It was demonstrated that Hibiscus protocatechuic acid has a protective effect against cytotoxicity and genotoxicity induced by tert-butylhydroperoxide in a primary culture of rat hepatocytes (Milletti M et al., (1959)) and it was proposed that one of the mechanisms of this

protective effect was associated with the scavenging of free radicals. Hibiscus protocatechuic acid also inhibits lipopolysaccharide-induced rat hepatic damage (Chewonarin T *et al.*, (1999)) and tert-butylhydroperoxide-induced rat hepatotoxicity (Tseng TH *et al.*, (1997)). 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 (Liu CL *et al.*, (2002)) 4-nitroquinoline-1-oxide in the oral cavity (Lin WL *et al.*, (2003)) , azoxymethane in the colon (Tanaka T *et al.* (1993)) ,N-methyl-N-nitrosourea in glandular stomach tissue (Tanaka T *et al.*, (1994)) and Nbutyl- N-(4-hydroxybutyl)nitrosamine in the bladder (Kawamori T *et al.*, (1994)). Topical application of Hibiscus protocatechuic acid prior to treatment with 12-O-tetradecanoylphorbol-13-acetate to female mice, initiated with benzo[α]pyrene, inhibited the incidence of **tumours**, also demonstrated that Hibiscus protocatechuic acid inhibits the survival of human promyelocytic HL-60 cells in a concentration- and time-dependent manner(Tanaka T *et al.*, (1995)), Hirose Y *et al.*, (1995)) . Hibiscus protocatechuic acid was also found to inhibit the oxidation of low-density lipoprotein, which increase the incidence of atherosclerosis, induced by either copper or a nitric acid donor (Tseng TH *et al.*, (1998)). The anthocyanins of *H. sabdariffa* were also shown to have a protective effect against tertbutylhydroperoxide-induced hepatic toxicity in rats. The anthocyanins were able to quench the free radicals of 1, 1-diphenyl-2-picrylhydrazyl and this antioxidant effect was also demonstrated by the ability of the anthocyanins to reduce the cytotoxicity induced by tert-butylhydroperoxide in rat primary hepatocytes and to attenuate hepatotoxicity in rats (Tseng TH *et al.*, (2000)). Administration of the anthocyanins isolated from the plant (100 or 200 mg/kg/day for 5 days) significantly reduced the activities of

the serum enzymes indicative of liver damage, ameliorated histological lesions and reduced oxidative liver damage. Similar dosages of *H. sabdariffa* anthocyanins were effective in significantly mitigating the pathotoxicity induced by paracetamol in mice (Lee M-J *et al.*, (2002)) . It has also been reported that anthocyanins protect against DNA damage induced by tert-butylhydroperoxide in rat smooth muscle and hepatoma cells (Wang CJ *et al.*, (2000)). In view of the established strong antioxidant and antilipid peroxidation actions of *H. sabdariffa* extracts and the compounds they contain , and because many diseases and conditions (for example, diabetes and aging) are thought to involve lipid peroxidation and the generation of free radicals (Lazze MC *et al.*, (2003)) (Suboh SM *et al.*, (2004)) , the anthocyanins and Hibiscus protocatechuic acid may potentially be useful in ameliorating or preventing these diseases and conditions.

2.1.4. 3. Antipyretic, antinociceptive and anti-inflammatory activities

An aqueous extract of *H. sabdariffa* was effective in inhibiting yeast-induced pyrexia in rats, and in reducing the reaction time in a hot plate, tail-flick assay, indicating that the extract has antipyretic and antinociceptive actions. The extract, however, was without significant effect in the rat paw carrageenan-induced oedema test , which is one marker used for assaying anti-inflammatory action. However, in one clinical trial involving 50 patients, administration of a decoction of dried fruit (3 g/person, three times every day for 7 days to 1 year) was shown to produce anti-inflammatory activity (Mohamed *et al.*, (2007)).

More work on this aspect, using several models for the assay of antiinflammatory activity is warranted. It was suggested that the above antipyretic and antinociceptive actions could be attributed to flavonoids,

polysaccharides and organic acids (Poon HF et al.,(2004)) Further work is required to study the effects of fractions and isolated compounds in experimental antiinflammatory, antipyretic and antinociceptive tests and their possible mechanism(s) of action.

2.1.4.4. Renal effects

Workers studied, in six normal Thai subjects, the changes in urine composition that follow the consumption of *H. sabdariffa* extract at different concentrations and for various periods of time (Vincent AM et al.,(2004)). This work indicated that consumption of *H. sabdariffa* extract resulted in significant decreases in the urinary concentrations of creatinine, uric acid, citrate, tartrate, calcium, sodium, potassium and phosphate, but not oxalate. It was noted that the low dose of *H. sabdariffa* (16 g/day) caused a more significant decrease in salt output in the urine than a high dose (24 g/day). A significant uricosuric action was noted in rats given a decoction of the dried calyx at an oral dose of 1 g/kg Dafallah AA et al., (1996)), (Kirdpon S et al., (1994)) .

2.1.4.5. Anticholesterol effects

H. sabdariffa calyx (5% or 10%) was fed to rats with hypercholesterolaemia for 9 weeks (Caceres A et al., (1987)). The treatment progressively lowered the different lipid fractions in plasma, heart, brain, kidney and liver; and also decreased the activities of several plasma enzymes used in tests as markers of tissue function. This treatment, however, slightly raised the content of plasma phospholipids. Although the mechanism of action of *H. sabdariffa* as a cholesterol-lowering agent was not elucidated in this work, it was hypothesized, albeit with no experimental evidence, that the extract may contain some compounds that activate hormonal secretions, such as adrenocortical hormones, which stimulate the metabolic pathway of cholesterol by

conversion into other compounds. The anticholesterol action of *H. sabdariffa* (0.5% or 1%) was confirmed in rabbits fed cholesterol for 10 weeks. This treatment was effective in reducing the serum concentrations of triglycerides, total cholesterol and low-density lipoprotein cholesterol, and in mitigating atherosclerosis in the aorta. Histopathologically, it was found that feeding *H. sabdariffa* had reduced foam cell formation and inhibited smooth muscle cell migration and calcification in the blood vessel of treated rabbits (Mojiminiyi FBO et al., (2000)) .

2.1.4.6. Antibacterial, antifungal and antiparasitic actions

Ethanollic extract of the dried leaves was found to be ineffective against *Lumbricus terrestris* (El-Saadany SS et al., (1991)) . An aqueous extract of dried sepals of *H. sabdariffa* was active against *Schistosoma mansoni*.

2.1.4.7. Interaction with drugs

The interaction of three Sudanese beverages, including *H. sabdariffa*, with the kinetics of chloroquine was studied in human volunteers (Chen C-C et al., (2003)). *H. sabdariffa* was found not to have a significant effect on any pharmacokinetic parameter, indicating its safety when taken with drugs that may have the metabolic pathways of chloroquine. More recently study showed that the effect of *H. sabdariffa* water extract on paracetamol (acetaminophen) kinetics in healthy men (Boum B et al. (1985)). On the whole, the administration of the extract induced no significant changes in the major kinetic parameters of paracetamol, although very minor and probably biologically insignificant alterations in some parameters were observed. In view of the fact that *H. sabdariffa* drinks may be ingested with medicines, more studies to ascertain the presence or absence of interactions with drugs of different metabolic profiles are warranted.

2.1.5. Chemical composition

Phytochemicals are aromatic substances synthesized by plants which in many cases have been suggested to be an inherent defense mechanism to microbial infection, insect and herbivore predation. Phenolics are one of the groups of phytochemicals which comprise simple phenols, phenolic acids, quinones, flavonoids, flavones, flavonols, tannins and coumarins. Aqueous extracts of *H. sabdariffa* L calyces have been analyzed and the results attribute the bright red color to the presence of anthocyanins, allowing the extract to fit in a phenolic and flavonoid profile. Eighty-five percent of these anthocyanins was identified as delphinidin 3-sambubioside and cyaniding 3-sambubioside. (Moreover, Badreldin and others (2005)) examined the chemical composition of aqueous extracts of *H. sabdariffa* L and reported the presence of hibiscitrin, gossypitrin, sabdaritrin, flavonol glycosides and some organic acids such as citric, malic, tartaric and ascorbic (Cowan 1999; Tsai and others 2002).

2.2. Protocatechuic acid

Protocatechuic acid (PCA) is a type of widely distributed naturally occurring phenolic acid. PCA has structural similarity with gallic acid, caffeic acid, vanillic acid, and syringic acid which are well-known antioxidant compounds.

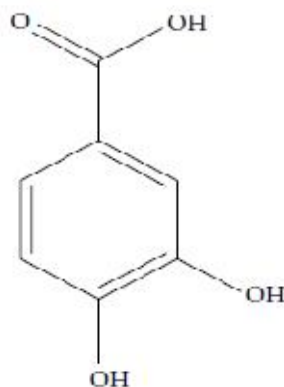


Fig. 2.1 Chemical structure of protocatechuic acid

2.2.1. Distribution and Occurrence

Protocatechuic acid occurs in pigmented onion scales *Allium cepa* (P. Vitaglione, *et al.*, (2010)) which enables them resist onion smudge, a fungal disease due to *Colletotrichum circinans*. Hibiscus protocatechuic acid (PCA) is a simple phenolic compound isolated from the dried flowers of *Hibiscus sabdariffa* L. (C.-Y. Chao and M.-C. Yin *et al.*, (2009)), a Chinese herbal medicine, which is reported to be antiseptic). (wikipedia), aphrodisiac, astringent, cholagogue, demulcent, digestive, diuretic, emollient, purgative (N. Mahadevan *et al.*, (2010)), refrigerant, resolvent, sedative, stomachic, and tonic. Also this is a folk remedy for abscesses, bilious conditions, cancer, cough, debility, dyspepsia, dysuria, fever, hangover, heart ailments, hypertension (C. C.-C. Hsieh *et al.*, (2006)), neurosis, scurvy, and strangury. Protocatechuic acid is also found in Carrot (*Daucus carota*) and in mushrooms such as *Agaricus bisporus* (White Button Mushroom) or *Phellinus linteus* and has shown good chemopreventive properties. Protocatechuic acid is considered as an active component of some traditional Chinese herbal medicines such as *Cibotium barometz* (L.) (X.

Li *et al.*,(2011)) *J.S, Stenoloma chusanum* (L.) Ching, *Ilex chinensis* Sims. Fruits of *Ficus* species are rich source of polyphenolic compounds and flavanoids which are responsible for strong antioxidant properties that help in prevention and therapy of various oxidative stress related diseases such as neurodegenerative and hepatic diseases. Acai oil, obtained from the fruit of the Aca'i palm (*Euterpe oleracea*) (L. A. Pacheco-Palencia *et al.*,(2008)), is rich in protocatechuic acid (630 ± 36 mg/kg). Acai oil has a relatively high content of polyphenols, which in turn has been linked to a range of reported (mostly *in vitro*) antioxidant, anti-inflammatory, antiproliferative, and cardioprotective properties. PCA also occurs in rich quantity in various multiple fruits such as berries (raspberry, blueberry, mulberry, strawberry, cranberry, and gooseberry). It is also known to occur in Loquat fruit, wine, honey, and soybean.

2.2.2. Chemical Properties

PCA is chemically known as 3,4-dihydroxybenzoic acid. It is a phenolic compound naturally occurring in various plant species. Phenolic compounds are considered secondary metabolites and are derived from phenylalanine via the shikimic acid pathway. Phenolics possess an aromatic ring and have one or more hydroxyl groups. Plants contain a large variety of phenolic derivatives, including benzoic acids, cinnamic acid derivatives, flavonoids, isoflavonoids, lignans, and tannins. In plants the main phenolic classes are hydroxyl benzoic acids, hydroxycinnamic acids, flavonols, anthocyanins, flavan-3-ols and proanthocyanidins, and ellagitannins.

2.2.3. Physical Properties

PCA is a gray to tan solid crystalline powder, with a 221°C melting point and 410°C boiling point at 760mm Hg. It has a mild phenolic odour. It is

sparingly soluble in water (1 :50), soluble in alcohol, ether and discolors in air. PCA is generally stable but incompatible with strong oxidizing agents and strong bases. It irritates lungs, eyes, and skin.

2.2.4. Pharmacological Properties

A variety of research work has been carried out on protocatechuic acid, its derivatives, and coforms (esters, aldehydes, etc.). It has been found useful for treatment and/or prophylaxis for a large number of various ailments associated with oxidative stress damage in multiple body systems *in vitro* and *in vivo*.

2.2.4.1. Antibacterial Activity

Roselle calyx (*Hibiscus Sabdariffa* L.) extract and protocatechuic acid were both found to decrease lipid oxidation levels in ground beef tissue. Protocatechuic acid also exhibited dose-dependent effect. The addition of roselle calyx extract or protocatechuic acid did not affect cooking loss, pH value, sensory attributes and content of fat, protein, and moisture of beef samples during storage at 4°C for 15 days. Roselle calyx also shows the presence of protocatechuic acid. These data support that roselle calyx extract and protocatechuic acid may be used for muscle foods to prevent contamination from campylobacter and aerobes and delay lipid oxidation and also serve as a good food preservative (C.-L. Liu *et al.*, (2002)).

2.2.4.2. Antioxidant Activity

Protocatechuic acid and other structurally similar dihydroxy and trihydroxyphenolic acids, acid, gallic acid, 3,4,5-trihydroxyphenylacetic acid, 3-(3,4,5-trihydroxyphenyl) propanoic acid, and 3-(3,4,5-trihydroxyphenyl) propanoic acid were examined for their total

antioxidant capacity. 3, 4, 5-trihydroxy-phenylacetic acid showed to be most potent radical scavenger generated by AAPH in liposomes. In the lipid peroxidation assay 3, 4-dihydroxyphenylacetic acid was observed to be the most effective compound. The antioxidant activities of protocatechuic acid. were measured *in vitro* using various antioxidant assays including 1,1-diphenyl-2-picryl-hydrazyl (DPPH \cdot), 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS $^{+}$), superoxide anion radicals (O $^{2-}$) and hydroxyl radical (\cdot OH) scavenging activity, ferric ions (Fe $^{3+}$) and cupric ions (Cu $^{2+}$) reducing power, ferrous ions (Fe $^{2+}$), and cupric ions (Cu $^{2+}$) chelating activity, compared with the positive controls Trolox or BHT. PCA along with positive controls exhibited dose-dependently antioxidant ability. Comparing to a standard antioxidant Trolox, the relative antioxidant activity of PCA (i.e., the ratio of IC $_{50}$ (Trolox)/IC $_{50}$ (PCA)) was calculated as 2.8, 2.3, 3.7, 6.1, 4.2, 1.0, 2.7, and 1.5, respectively, for DPPH, ABTS, reducing power (Fe $^{3+}$), reducing power (Cu $^{2+}$), superoxide anion radical-scavenging, hydroxyl radical-scavenging, chelating ability (Fe $^{2+}$), and chelating ability (Cu $^{2+}$). Comparing to Trolox, PCA shows much more effective antioxidant activity *in vitro* in both lipid and aqueous media. Hence, it could therefore be used in pharmacological or food industry as a natural antioxidant

It may exhibit antioxidant activity by both chelating metal transition ions as well as by scavenging free radicals via donating hydrogen atom (H \cdot) or electron (e). Protocatechuic acid PCA is considered as an active component of some traditional Chinese herbal medicines such as *Cibotium barometz* (L.) (X. Li *et al.*, (2011)).

reported to possess various pharmacological effects which may be closely correlated with its antioxidant activities. Hibiscus PCA supplementation

was found to be beneficial in enhancing antioxidant status and inhibiting oxidative stress induced by exhaustive exercise in skeletal muscles.

2.2.4.3. Antidiabetic Activity

Protocatechuic acid at 1% and 2% when given to d-galactose treated mice for 8 weeks decreased reactive oxygen species levels, protein carbonyl, carboxymethyllysine, pentosidine, sorbitol, fructose, and methylglyoxal. PCA also shows anti-inflammatory properties in this regard by decreased release of interleukin (IL)-1 beta, tumor necrosis factor-alpha, and prostaglandin E₂ in brain. Protocatechuic acid might be helpful for the prevention or alleviation of ageing due to prevention of brain inflammatory and glycation injury. PCA at 2% or 4% when supplied to diabetic mice for 12 weeks was useful in preventing glycation associated diabetic complications. In other study cyanidin-3-O- β -glucoside (B. Scazzocchio *et al.*, (2011)) and PCA have been proposed to exert insulin-like activities by PPAP γ activation, evidencing a causal relationship between this transcription factor and adiponectin and GLUT4 up regulation. Thus PCA may be a promising antidiabetic agent for the future.

2.2.4.4. Anticancer Activity

Protocatechuic acid seems to have chemopreventive potential because it inhibits the *in vitro* chemical carcinogenesis and exerts proapoptotic and antiproliferative effects in different tissues (T. Tanaka *et al.*, (2011)). The mechanism of the chemopreventive action of protocatechuic acid is mostly associated with antioxidant activity, including inhibition of generation as well as scavenging of free radicals and up regulating antioxidant enzymes. It influences phases 1 and 2 of the metabolism of certain carcinogens and, perhaps directly blocks specific binding sites of

ultimate carcinogens with DNA molecule, thus preventing adduct formation that may result in mutations and neoplastic transformation. Other biological aspects seem to have influence on the activity of inducible isoenzyme of cyclooxygenase and nitric oxide synthase, cell cycle regulating proteins, or inflammatory cytokines, which are involved in oncogenesis. Thus PCA seems to have potential cancer chemo preventive properties.

2.2.4.5. Antiulcer Activity

Protocatechuic acid ethyl ester was studied in rats in which gastric ulcers were induced by oral administration of ethanol or aspirin or by pyloric ligation (K. J. Kore *et al.*, (2011)). PCA ethyl ester administered at the dose of (30mg/kg and 60mg/kg i.p) 30min prior to ulcer induction was found to possess significant antiulcer property and the ulcer index was significantly less in comparison to vehicle control animals. The mechanism of action of PCA ethyl ester may be due to either cytoprotective action of the drug or by Strengthening the gastric mucosa thereby enhancing mucosal defense. Similarly protocatechuic acid may also possess a certain level of antiulcer properties (K. J. Kore *et al.*,(2011)).

2.2.4.6. Antiageing Activity

Protocatechuic acid derived from the dried fruits of *Alpinia oxyphylla* has proved to be a potential antiageing compound on spleen and liver antioxidant system in aged rats (G.-F. Shi *et al.*, (2006)). Young and old rats were treated with single doses of Alpinia PCA (5mg/kg (low dose) or 10mg/kg (high dose) i.p. for 7 days). The results proved that Alpinia PCA significantly elevated the splenic weights, increased the activities of glutathione peroxidase and catalases, and decreased the malondialdehyde

level of aged rats. Thus PCA may be therapeutically utilized to minimize age-associated disorders where oxidative damage is the major cause (G.-F. Shi *et al.*, (2006)).

2.2.4.7. Antifibrotic Activity

Studies have shown protocatechuic aldehyde to possess beneficial antifibrogenic effects. Transforming growth factor- β 1 (TGF- β 1) and connective transforming growth factor (CTGF) are associated with the pathophysiology of liver fibrosis. In carbon tetrachloride (CCL₄) induced rat liver fibrosis model, liver fibrosis grade, and histopathological changes were evaluated, and biochemical indicators were determined. Protocatechuic aldehyde was seen to inhibit the levels of TGF- β 1, CTGF inhibit HSCs proliferation, type I collagen, and type III collagen in TNF- α stimulated HSCs. Also it causes significant reduction in fibrosis grade, ameliorates biochemical indicators, and histopathological morphology and reduces liver TGF- β 1 and CTGF expression. (C. Li *et al.*, (2012)).

2.2.4.8 Antiviral Activity

Protocatechuic aldehyde derived from the Chinese herb, *Salviamiltiorrhiza*, has been reported to inhibit hepatitis B virus (HBV) replication in HepG2 2.2.15 cell line *in vitro* and duck hepatitis B virus (DHBV) replication in duckling's *in vivo*. Protocatechuic aldehyde's mechanism seemed to down regulate the secretion of HBsAg and HBeAg and decrease the release of HBV DNA from HepG2 2.2.15 in a dose- and time-dependent manner occurring at concentrations between 24 and 48 g/ml. Also protocatechuic aldehyde when given (25, 50, or 100mg/kg, i.p. twice daily) also reduced viremia in DHBV-infected ducks. This activity tells us that structurally similar compound protocatechuic acid may also

possess certain levels of antiviral activity and can be an effective antiviral agent (Z. Zhou *et al.*, (2007))

2.2.4.9. Anti-Inflammatory, Analgesic and Antiseptic Properties

Protocatechuic acid has shown promising anti-inflammatory and analgesic activity in different rat models (carrageenan-induced paw oedema, cotton pellet-induced granuloma, and Freund's adjuvant arthritis) (A. B. Lende *et al.*, (2011)), of inflammation and chemical and heat induced mouse models of pain. Treatment with PCA inhibits significantly different biological parameters like hind paw oedema, granuloma exudates formation, and arthritis index in carrageenan oedema, cotton pellet granuloma, and Freund's adjuvant arthritis, respectively. The biochemical parameters like glutathione, superoxide dismutase, catalase, lipid peroxidation and NO in oedematous or in liver tissues and serum alanine aminotransferase, and lactic dehydrogenase occurring during different types of inflammation were either significantly restored or inhibited with PCA pretreatment. Reference (. R. H. Liu *et al.*, (2004)) in other study anthocyanins and their breakdown metabolites, protocatechuic, syringic, gallic, and vanillic acids were evaluated on different parameters involved in atherosclerosis, including inflammation, cell adhesion, chemotaxis, endothelial function, estrogenic/antiestrogenic activity and angiotensin-converting enzyme (ACE) inhibitor activity. Protocatechuic acid was found to exhibit a slight inhibitory effect on NO production and TNF- α secretion in LPS-INF- γ -induced macrophages. All anthocyanins showed an ACE-inhibitory activity. PCA has also displayed its anticoagulant, anti-inflammatory, and antioxidative effects in diabetic mice. PCA at 1%, 2%, and 4% was given to diabetic mice for 8 weeks which significantly lowered plasma glucose and increased insulin levels. Also PCA treatments at 2% and 4% significantly lowered plasminogen

activator inhibitor-1 activity and fibrinogen level; increased plasma activity of antithrombin- III and protein C; decreased triglyceride content in plasma, heart, and liver; elevated glutathione level and the retention of glutathione peroxidase and catalase activities in heart and kidney. PCA treatments also reduced the levels of interleukin- 6 and tumor necrosis factor- α in heart and kidney. Thus PCA could be highly useful in diabetic complications via its triglyceride-lowering, anticoagulatory, antioxidative, and anti-inflammatory effects (A. B. Lende *et al.*, (2011)).

2.2.4.10. Antiatherosclerotic and Hyperlipidemic Activities

Protocatechuic acid (PCA) has been found to possess the antiatherosclerotic effect. PCA inhibits monocyte adhesion to tumor necrosis factor- α (TNF- α)-activated mouse aortic endothelial cells, which is associated with the inhibition of vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) expression and reduces NF- κ B binding activity.

Protocatechuic possesses the antiatherogenic effect by virtue of its anti-inflammatory activity. Protocatechuic aldehyde (PA), isolated from the aqueous extract of the root of *Salvia miltiorrhiza*, an herb used in traditional Chinese medicine which has been used to treat a variety of vascular diseases, was tested on the migration and proliferation of VSMCs and platelets due to platelet derived growth factor (PDGF). DNA 5-bromo-2-deoxyuridine (BrdU) incorporation and wound-healing assays indicated that PA significantly attenuated PDGF-induced proliferation and migration of VSMCs at a pharmacologically relevant concentration (100 μ M). On a molecular level, it was observed down regulation of the phosphatidylinositol 3-kinase (PI3K)/Akt and the mitogen-activated

protein kinase (MAPK) pathways, both of which regulate key enzymes associated with migration and proliferation.

The ethanolic polyphenolic extracts of *H. Sabdariffa* possess significant antioxidant and hyperlipidemic activities. They have shown promising effects on decrease of serum total cholesterol, VLDL-C, LDL-C, LDL-C:HDL-C risk ratio, and atherogenic index in rats. Thus PCA which is a rich polyphenolic constituent of *H. sabdariffa* may serve as a good hyperlipidemic agent (A. R. Borate *et al.*, (2011)).

2.4.4.11. Cardiac Activity

Protocatechuic acid present in the aqueous extract of petals of *Hibiscus sabdariffa* exhibited antihypertensive and cardioprotective effects in established stages of 2-Kidney, 1-Clip renovascular hypertension model

in rats. This study supports the traditional use of *Hibiscus sabdariffa* exhibiting antihypertensive and cardioprotective effects and may be a useful antihypertensive agent. *Salvia miltiorrhiza* (A. R. Borate *et al.*, (2011)).has long been used in the traditional Chinese formulations for the treatment of heart ischemic diseases. Protocatechuic acid is its major chemical constituent. Isoproterenol induced acute myocardial infarction in rats showed positive treatment effects with the extracts of *Salvia miltiorrhiza* (L.Zhou,*et al.*,(2005)) (29.76 or 59.52mg/kg). Isoproterenol-treated rats showed reductions in left ventricular systolic pressure as well as in maximum and minimum rate of developed left ventricular pressure, together with an increase in left ventricular end-diastolic pressure. Also an increase in serum levels of lactate dehydrogenase, glutamic oxaloacetic transaminase, creatine kinase, and malondialdehyde was seen and decrease in serum activities of glutathione peroxidase and superoxide dismutase was observed. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)

(R.Zhou *et al.*,(2012)) cardiotoxicity in 3-4 months old rats was studied and protocatechuic acid treatment at the dose of 100mg/kg for 45 days was found to decrease the levels of TBARS, while increasing those of glutathione, catalase, glutathione peroxidase, and superoxide dismutase. Also PCA prevented histopathological alterations such as necrosis and hemorrhage in heart tissue induced by TCDD.

2.4.4.12. Hepatoprotective Activity

Hibiscus protocatechuic acid (PCA), a simple isolated from *Hibiscus sabdariffa* L. was found to be protective against oxidative damage induced by tert-butylhydroperoxide (t-BHP) in a primary culture of rat hepatocytes due to its antioxidant mechanism of action. *Hibiscus sabdariffa* L. PCA possesses free radical-scavenging capacity and protects against oxidative damage induced by tert-butylhydroperoxide (t-BHP) in rat primary hepatocytes. PCA (50–100mg/kg) by gavage for 5 days inhibited t-BHP-induced tyrosine phosphorylation in the liver and was found to be effective against t-BHP-induced hepatotoxicity by means of its antioxidant and anti-inflammatory characteristics accompanied by blocking of stress signal transduction. Alpinia PCA isolated from the dried fruits of *Alpinia oxyphylla* Miq. at the doses of 5–10mg/kg (i.p.) for 7 days in young and old rats was found to possess antiageing effects. It significantly elevated the splenic weights, increased the activities of GSH-PX and CAT, and decreased the MDA level of aged rats. Thus PCA was thought to be therapeutically useful to minimize age-associated disorders where oxidative damage is the major cause (O. Ciftci, *et al.*, (2013)).

2.4.4.13 Nephroprotective Activity

The aqueous extracts from *Hibiscus sabdariffa* richly comprising protocatechuic acid possess a potent protective effect against the oxidative stress induced by sublethal dose of Malathion (an organophosphorus pesticide on the adult male rat kidney). Aqueous extract in a daily dose of 500mg/kg b.wt./day decreased the oxidative stress levels, prevented cellular degeneration and necrosis of the renal tissues. Also serum urea and creatinine were decreased and GSH and SOD levels were also increased significantly. This study proves the utility of PCA in preventing damage by agents causing oxidative stress mediated nephrotoxicity. *Rhus verniciflua* Stokes (RVS) containing flavonoids have antioxidant effects. Protocatechuic acid is also a major phenolic acid present in *Rhus verniciflua* Stokes. The cytotoxic and nephroprotective effects of RVS were evaluated *in vitro* in cisplatin treated Madin—Darby Canine kidney (MDCK)-I renal cells. Also its *in vivo* effects were studied in BALB/c mice inoculated with CT-26 colon adenocarcinoma cells and treated with cisplatin. RVS prevented cisplatin-induced cytotoxicity and ROS release against MDCK-I cells.

RVS also exerted significant antitumor activity against CT- 26 cells. The serum and kidney parameters were improved, which suggests that protocatechuic acid present in RVS can be isolated and usefully applied to the neoplastic patients as a combined chemopreventive agent with cisplatin. PCA is structurally 3,4-dihydroxy benzoic acid and its structurally similar analogue and antioxidant 2,3- dihydroxybenzoic acid (DHB) reverses the vancomycin-induced nephrotoxicity in rats. Vancomycin-induced nephrotoxicity involves oxidative injury due to free radical formation. It can be suggested that PCA could also show the same efficacy in prevention of nephrotoxicity similarly (J.H. Lee, *et al* (2009)).

2.2.4.14. Neurological Effects

Protocatechuic acid (PCA) isolated from the kernels of *Alpinia oxyphylla* protected from oxidative stress induced neurotoxicity due to hydrogen peroxide apoptosis in cultured PC12 cells. It was also found to play crucial role in the proliferation and neuroprotection of cultured neural stem and progenitor cells. PCA induced neuronal maturation and efficiently promoted neurite outgrowth. Protocatechuic acid also showed positive effects on PC12 cells treated with MPP (+) by inhibition of the oligomerization of alpha-synuclein which affects neuronal viability. PCA inhibited the cytotoxicity, apoptotic morphology, reduction of TH expression, and abnormal oligomerization of alpha-synuclein in PC12 cells. MPP+ (1-methyl- 4-phenylpyridinium ion) has also been found to cause apoptosis in dopaminergic PC12 cells. Protocatechuic acid present in ethyl acetate extract of *Alpinia oxyphylla* was found to possess neuroprotective activity against 1 methyl- 4-phenylpyridinium ion (MPP+) induced apoptosis and oxidative stress in cultured PC12 cells in a dose-dependent manner. Thus PCA may find a valuable use in management of Parkinson's disease. The neurotrophic effects of protocatechuic acid on neurite outgrowth and survival in cultured newborn rat cerebral cortical neurons was determined and it was found out that PCA increased the number of survival neurons with neuritis and the average length of neuritis. Protocatechuic acid methyl ester isolated from fraction of the bark of *Machilus thunbergii* Sieb. et Zucc. (Lauraceae) was found to possess significant neuroprotective activities against glutamate-induced neurotoxicity in primary cultures of rat cortical cells at concentrations ranging from 0.1 micro M to 10.0 micro M and were comparable to MK-801 which is a well known inhibitor of glutamate receptor (S. G. Guan, *et al*(2006)).

2.2.4.15. Effects on Reproductive System

Protocatechuic acid (1mg/kg, p.o. for 45 days) administration in rats was found to be highly beneficial in protecting against reproductive toxicity caused by 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD), an environmental contaminant. TCDD (2 ug/kg per week) caused oxidative stress damage via an increase in the levels of TBARS and decrease in the levels of glutathione, catalase, and SOD. It also caused testicular damage, decreased the serum testosterone levels, and reduced the sperm motility and sperm count. Treatment with PCA caused a significant reversal of the oxidative, hormonal, histopathological, and spermatological parameters (Beytur, *et al*(2002)).

3. Materials and Methods

3.1. Materials

3.1.1. Plant material

The plant material has been bought from local market in Khartoum North, Sudan.

3.1.2. Chemicals

- Ethanol 96%
- Diethyl ether
- Hydrochloric acid
- Lead acetate
- Ferrous sulphide
- Activated charcoal (decolorized)

3.1.3. Apparatus and Equipments

- Round bottom flask
- Separatory funnel
- Soxhlet Extractor
- Rotary evaporator
- FTIR-8400S- Shimadzu

3.2. Methods

3.2.1. Isolation of Protocatechuic acid

The ground plant material has been exhaustively extracted with Soxhlet extractor using 96% ethanol. The extract was filtered and concentrated under reduced pressure into thick syrup, sufficient distilled water was added and filtered; the filtrate was decolorized using activated charcoal. To the result solution; an aqueous solution of lead acetate was added in

portions and stirring while it was boiling, a white precipitate formed has been washed several time and then filtered. The precipitate then suspended in distilled water and H₂S gas has been passed through suspension till it completely converted into black precipitate then filtered, the filtrate completely concentrated under reduced pressure. The crude extract was extracted with diethyl ether under reflux. The ether extract was concentrated to minimum volume and left overnight in refrigerator till white crystal has been formed.

3.2.2. Infra-red Spectroscopy

The potassium bromide dispersion of protocatechuic acid was prepared and the complete spectrum was scanned against blank disk. The absorption and vibration bands were observed at various frequencies using FTIR-8400S- Shimadzu.

3.2.3. Antimicrobial activity

3.2.3.1. Preparation of bacteria suspension

One cm³ aliquots of a 24 hours broth culture of the test organism were aseptically distributed onto nutrient agar slopes and incubated at 37⁰C for 24 hours.

The bacteria growth was harvested and washed off with sterile normal saline, and finally suspended in 100 cm³ of normal saline to produce a suspension containing about (10⁸-10⁹) Colony forming units per cm³, and the suspension was stored in the refrigerator at 4 ⁰C until used. The average number of viable organisms per 1cm³ of the stock suspension was determined by means of the surface viable counting technique (Miles A A & Misra S S., (1993)).

Several dilutions of the stock suspension were made in sterile normal saline in tubes and 0.02cm³ volumes (one drop) of the appropriate dilutions were transferred by transfer pipette adjustable volume automatic- titer pipette onto the surface of dried nutrient agar plates.

The plates were allowed to stand for 2 hours at room temperature for the drops to dry and then incubated at 37 °C for 24 hours. After incubations, the number of Colonies per drop (0.02cm³) was multiplied by 50 and the dilution factor to give the viable count of stock suspension expressed as the number of Colony forming units. (C.F.U. per cm³ of suspension).

Each time a fresh stock suspension was prepared and all the above experimental conditions were maintained (constant) so that suspension with very close viable counts would be obtained.

3.2.3.2. Preparation of Fungal Suspensions

The Fungal cultures were maintained on sabourand dextrose, incubated at 25 °C for days. The fungal growth was harvested and washed with sterile normal saline and finally suspended in 100cm³, of sterile normal Saline, and the suspension was stored in the refrigerator until used.

3.2.3.3. Preparation of the media

28.0g Nutrient agar and 62.0g of Sabourud dextrose agar were dissolved separately each in one liter of distilled water, sterilized by autoclave at 121°C.

3.2.3.4. Test organisms

Standard strains of the test organisms were obtained from the American culture type collection (ATCC), the following organisms were used:

i- *Escherichia coli* ATCC25922 (gram positive).

ii- *Pseudomonas aeruginosa* ATCC27853(gram positive).

iii- *Staphylococcus aureus* ATCC25923(gram negative).

iv- *Bacillus subtilis* NCTC8236 (gram negative).

Two species of fungal, commonly causing systemic infections in immunocompromised patients were used are:

i- *Candida albicans* ATCC7596.

ii- *Aspergillus niger* ATCC9763.

4. Results and Discussion

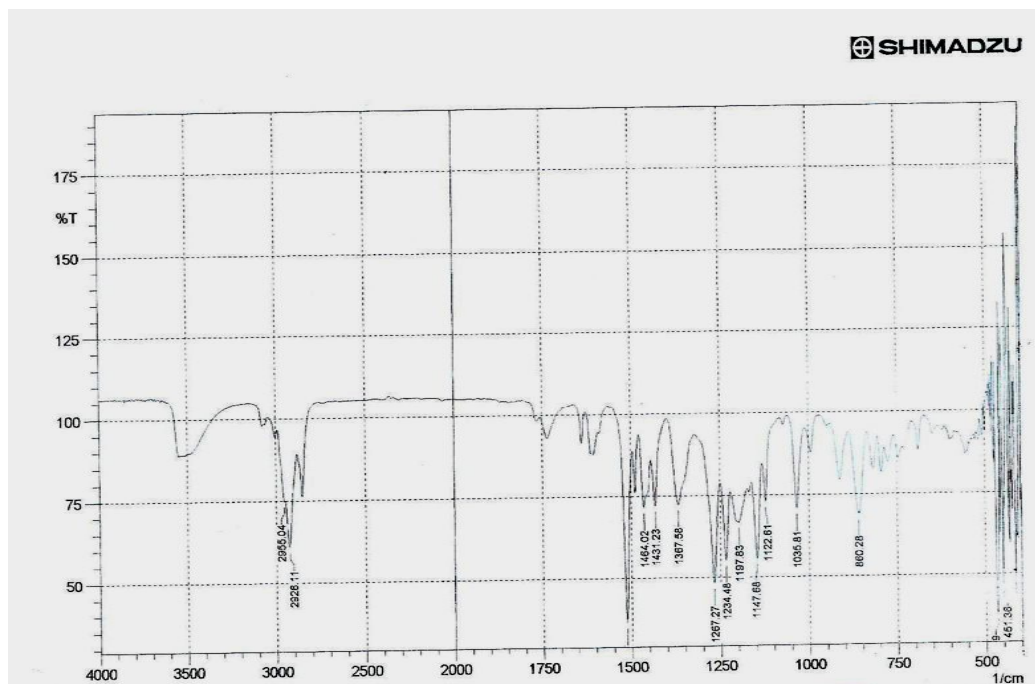
4.1. Isolation of protocatechuic acid

Although protocatechuic acid is widely distributed in plants as a constituent of many aromatic compounds, in the catechol tannins, in numerous resins and wood gums, in lignified wood, and as a constituent of various flavone and anthocyan pigments ((Abderhalden, et al., (1911))p. 1297), ((Czapek, et al.,(1921)) p. 479), ((Meyer, et al., (1923)), p. 641), its occurrence in the free state has been reported only in a few cases. Perkin (Perkin, et al., (1909)) reported its occurrence in the flowers of *Hibiscus sabdarifa*, a red sorrel of the West Indies, and in the flowers of *Thespesia lampas*, a small bush common to tropical jungles of India, Burma, and Ceylon. Free protocatechuic acid has also been reported present in the fruits of *Illicium anisatum*, the Chinese anise, by Eykman ((Wehmer.,et al.,(1911)) p. 214) and in the leaves of the wine grape, *Vitis vinifera*, by Boettinger ((Wehmer.,et al.,(1911)) p. 213).

It is of sufficient importance to make note here that the flavonol quercetin was also present in the two plants from which Perkin (Perkin, et al., (1909)) isolated protocatechuic acid. The isolation of protocatechuic acid from pigmented onion scales thus represents the third instance in which the acid has been found associated with the flavonol quercetin. Perkin and Hummel (Perkin, et al.,(1896)) had also reported the isolation of quercetin from pigmented onion scales. Upon alkaline fusion the pigment quercetin breaks up into phloroglucinol, oxalic acid, and protocatechuic acid.

4.1.1 FTIR of protocatechuic acid

The IR (KBr disc) spectrum of protocatechuic acid (Fig.4.1.2) showed 3500 (OH). 2955.04, 2926.11 (C-H,Aliph.). 1700 (COOH). 1454.02, 1431.23, 1367.58 (C=C, Ar), 804,678 cm^{-1} (C-H, Ar, bending).



FTIR of protocatechuic acid

4.2. Antimicrobial activity

Two gram-negative and two gram-positive bacterial strains, representing common human bacterial pathogens were used for the antibacterial screenings. The gram-negative bacteria were *Escherichia coli* and *Pseudomonas aeruginosa*; the gram-positive were *Staphylococcus aureus* and *Bacillus subtilis* (Ibrahim Ibnusaud, et al.,(2002)). Two species of fungal, commonly causing systemic infections in immunocompromised patients were used are *Candida albicans* and *Aspergillus niger*.

The antibacterial and antifungal activity protocatechuic acid are shown in table 4.2.

Table 4.2. Antimicrobial activities of the protocatechuic acid

Conc.	Diameter of inhibition zone (mm)					
	<i>E.coli</i>	<i>P.aeruginosa</i>	<i>S. aureus</i>	<i>B. subtilis</i>	<i>C.albicans</i>	<i>A. niger</i>
10 %	10.11	13.14	9.10	11.12	-	-

The activity of the protocatechuic acid against both Gram-positive organisms may be indicative of the presence of broad spectrum antibiotic of title compounds. *S. aureus* is a Gram-positive spherical bacterium which is one of the most important etiological agents responsible for healthcare-associated infections. It is one of the main causes of hospital - and community-acquired infections which can result in serious consequences, its infections affect the bloodstream, skin, soft tissues and lower respiratory tracts. *S. aureus* can be a cause of central venous catheter-associated bacteremia and ventilator-assisted pneumonia. It also causes serious deep-seated infections, such as endocarditis and osteomyelitis (Olaleye, et al., (2007)). In addition to the infections listed above, *S. aureus* is often responsible for toxin-mediated diseases, such as toxic shock syndrome, scalded skin syndrome and staphylococcal food borne diseases (SFD). Hospitalized patients are particularly exposed to *S. aureus* infections due to their compromised immune system and frequent catheter insertions and injections (Morton JF (1987)).

These results suggest that of protocatechuic acid might be used in treating a wide range of pathogenic Gram-positive bacteria. Also protocatechuic acid was unable to inhibit *Candida albicans* and *Aspergillus niger* which implies that they could not be used to treat fungal diseases. Aqueous-methanolic extract of *H. sabdariffa* was found to exhibited antibacterial activities against *S.aureus*, *B. stearotherophilus*,

Micrococcus luteus, *Serratia marcescens*, *Clostridium sporogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Bacillus cereus*, *Pseudomonas fluorescens*, it is interesting to note that the *H. sabdariffa* extract was able to inhibit the growth of *E. coli* which was not sensitive to Streptomycin, a standard broad spectrum antibiotic. These antibacterial activities are likely due to the presence of the secondary metabolites present in the *H. sabdariffa* extract (Olaleye, et al., (2007)).

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