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DEPARTMENT OF CHEMISTRY AND CHEMICAL TECHNOLOGY

ISOLATION, CHARACTORISATION AND ELECTROCHEMISTRY OF GESHOIDIN FROM RHAMNUS PRINOIDES

BY MALATALIANA T. F

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SUPERVISORS: PROFESSOR ALEMAYEHU G. C.

: PROFESSOR ALEMU H.

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Declaration

This project has never been done in any previous fulfillment of the degree in BSc. Chemical Technology. The work in this document has been done by me with the help of my supervisors and all the sources of information have been approved by references.

Thabang Fernando Malataliana

Dedication

This project is dedicated to my family for the support they gave me to this extend and more importantly, to me for the effort I dedicated to myself for the success of this journey and sometimes with tremendous hardships of life and thanks to Almighty God I never gave up.

Thabang Fernando Malataliana

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Thabang Fernando Malataliana

Abstract

The leaves and the stems of Rhamnus Prinoides (Amharic name, Gesho and Sesotho name, Mofifi) are used as traditional medicine to treat pneumonia, gonorrhea, rheumatism, stomach-ache, as a gargle and so many more. In some countries such as Ethiopia, the leave and stems are used in traditional fermented beverages such as Tella and Tej. Chemical investigation on the leaves resulted in the isolation of a characteristic bitter principle known as naphthalenic compound β-*sorigenin-8-O*-β-*D-glucoside.* Then, the name Geshoidin was proposed for this novel glucoside. The structure of Geshoidin was established from the spectroscopic analysis of natural products.

The electrochemical behaviour of *Geshoidin* was investigated at a glassy carbon electrode in mixtures of citric acid and di-sodium hydrogen orthophosphate aqueous buffer system over a wide pH range (pH 2-11) using cyclic voltammetry. Chemically irreversible single oxidation and reduction peaks were obtained in the potential and pH region investigated. Variations in the peak potential and peak current of the oxidation peak have been observed as function of pH. The wave characteristics, the reversibility of the reactions, the diffusion coefficient and the number of electrons transferred have been studied. Linear sweep voltammetry was applied for the voltammetric determination of *Geshoidin* and a linear calibration curve over the range 1.00×10^{-6} - 1.00×10^{-4} mol dm^{-3} *Geshoidin* was achieved. The detection limit was found to be 5.00×10^{-7} mol dm^{-3} *Geshoidin*. For eight successive determinations of 1×10^{-5} mol dm^{-3} *Geshoidin*, a relative standard deviation (RSD) of 3.2% was obtained.

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1. INTRODUCTION

1.1. General.

The term “natural product” is commonly reserved for those organic compounds of natural origin that are unique to one organism, or common to a small number of closely related organisms. In most instances they appear to be non-essential to the plant, insect, or microorganism producing them, in marked contrast to the other organic compounds in nature, sugars, amino acids, nucleotides, and the polymers derived from them, which are both essential and ubiquitous [1].

Many higher plants produce economically important organic compounds such as oils, resins, tannins, natural rubber, gums, waxes, dyes, flavors and fragrances, pharmaceuticals, and pesticides. However, most species of higher plants have never been described, much less surveyed for chemical or biologically active constituents, and new sources of commercially valuable materials remain to be discovered. In the future, biologically active plant-derived chemicals can be expected to play an increasingly significant role in the commercial development of new products for regulating plant growth and for insect and weed control [2]. Many higher plants accumulate extractable organic substances in quantities sufficient to be economically useful as chemical feedstock or raw materials for various scientific, technological, and commercial applications. Natural substances are employed, either directly or indirectly, by a large number of industries, and natural plant products (phytochemicals) [1, 3]. Economically important plants serve as sources of industrial oils, resins, tannins, saponins, natural rubber, gums, waxes, dyes, pharmaceuticals, and many specialty products [4].

1.2. Classification of natural products.

For the sake of convenience, plant chemicals are often classified as either primary or secondary metabolites [1, 5]. (Proteins and nucleic acids are generally excluded from this classification). ***Primary metabolites*** are substances widely distributed in nature, occurring in one form or another in virtually all organisms. In higher plants such compounds are often concentrated in seeds and vegetative storage organs and are needed for physiological development because of their role in basic cell metabolism. As a general rule, primary metabolites obtained from higher plants for commercial use are high volume-low value bulk chemicals. They are mainly used as industrial raw materials, foods, or food additives and

include products such as vegetable oils, fatty acids (used for making soaps and detergents), and carbohydrates (for example, sucrose, starch, pectin, and cellulose). However, there are exceptions to this rule. For example, myoinositol and ß-carotene are expensive primary metabolites because their extraction, isolation, and purification are difficult [6].

Secondary metabolites on the other hand**,** are compounds biosynthetically derived from primary metabolites but more limited in distribution in the plant kingdom, being restricted to a particular taxonomic group (species, genus, family, or closely related group of families). Secondary compounds have no apparent function in a plant's primary metabolism but often have an ecological role; they are pollinator attractants, represent chemical adaptations to environmental stresses, or serve as chemical defenses against microorganisms, insects and higher predators, and even other plants (allelochemicals) [1, 6]. Secondary metabolites are frequently accumulated by plants in smaller quantities than are primary metabolites. In addition, secondary metabolites, in contrast to primary metabolites, tend to be synthesized in specialized cell types and at distinct developmental stages, making their extraction and purification difficult. As a result, secondary metabolites that are used commercially as biologically active compounds (pharmaceuticals, flavors, fragrances, and pesticides) are generally higher value-lower volume products than the primary metabolites. Thus, compared to primary metabolites (bulk chemicals); many secondary metabolites can be considered as specialty materials or fine chemicals [5, 6].

Examples of commercially useful plant secondary metabolites are nicotine, the pyrethrins, and rotenone, which are used in limited quantities as pesticides, certain steroids and alkaloids, which are used in drug manufacturing by the pharmaceutical industry. Secondary natural products often have highly complex structures with many chiral centers, which may determine biological activity; such complex compounds cannot be synthesized economically [5, 6]. A good example of a secondary metabolite having a high degree of structural complexity is the naturally occurring plant insecticide azadirachtin [6].

1.3. Characteristics of both the primary and secondary metabolites.

Economically important primary and secondary metabolites share several common characteristics. Most can be obtained from plant materials by steam distillation or by extraction with organic or aqueous solvents, and they tend to be relatively low in molecular

weight (generally less than 2000), excluding the biopolymers, natural rubber, condensed tannins, and the high molecular weight polysaccharides such as gums, pectin, and starch [5, 6].

As far as proteins are concerned, their extraction and use of specialized plant proteins from plant cells has limited importance for several reasons. First, their chemical structure imposes certain constraints on their use as biologically active compounds, that is, as drugs and pesticides. For example, most proteins cannot be absorbed readily through mammalian skin or insect exoskeleta, and most also cannot be administered orally (except to achieve local effect) because they are subject to digestive degradation by proteolytic enzymes [2].

1.4. Rhamnus Prinoides L 'Herit:

Rhamnus Prinoides L 'Herit, (Charles Louis 'Hérit, 15 June 1746 – 18 August 1800, conducted various studies of native trees and shrubs, also gaining interest in exotic flora), has a common name: *Dogwood*, Amharic name*: Gesho*, Sesotho name: *Mofifi* [1, 2, and 5].

The following figures represent Rhamnus Prinoides:

Figure 1: Growing Rhamnus Prinoides [7]

Figure 2: The leaves of Rhamnus Prinoides [7].

1.4.1 Plant description:

A. Taxonomic description

Current name: Rhamnus Prinoides L 'Herit, species name: Prinoides, (means an evergreen oak), genus name: Rhamnus, (an ancient Greek word (rhamnos) for blackthorn, meaning a "tuft of branches" and the family: Rhamnaceae.

B. Botanic description

Rhamnus Prinoides is a shrub sometimes with the tendency to scramble, or a small, dense, thick, bushy evergreen tree that may reach 6 - 9 m in height [7, 8, 9], usually spineless, with no buttresses; branchlets pubescent when young, glabrous when mature; bark grey to brown, smooth, with conspicuous lenticels, becoming dark brown with age. Flowers are greenish-yellow, small, pentamerous, inconspicuous, on slender stalks, in sparse auxiliary groups or clusters of 2-10, in the axils of the leaves; petals greenish; pedicels up to 1.9 cm long; ovary 3-4 celled. Fruits are berrylike (drupe), ovoid to almost circular, about 5 mm in diameter, shiny red becoming dark red when mature, sometimes almost black, usually clearly divided into 3 compartments, with a small saucer-shaped calyx, 3-seeded; stalk thin [10].

C. Ecology and distribution

R. Prinoides is widespread and locally common at medium to high altitudes, along water courses, in reverie forest and at the margins of evergreen forests. It shares this character with 2 other trees, Olea africana and Bequaertiodendron magalismontanum. The tree casts so deep a shade that it often prevents other growth around it. It can withstand a fair amount of frost and R. Prinoides grow readily in most soils, but thrives in moist, humus-rich soils [10, 11].

D. Geographic distribution

Native: Botswana, Eritrea, Ethiopia, Lesotho, Namibia, South Africa, Swaziland, Uganda

Exotic: Kenya.

1.4.2. Functional uses of the plant.

A. Services

As services, the plant is used as- erosion control: A good tree to plant along drainage lines or furrows to protect stream banks. Shade or shelter: R. Prinoides can be planted as a hedge along camp fences or along the edges of crops to act as a low windbreak. It may also be planted by fishponds to protect and shade the fish. Ornamental: The attractive leaves and berries make striking floral arrangements, and because it grows slowly and has a non-aggressive root system, it is perfect for a small garden. It is easily grown and can be trained in various bonsai styles. Boundary or barrier or support: R. Prinoides can be used effectively as a hedge or screen plant in the garden.

B. Products

As products, the fruits are used as food (edible) and are also used for the treatment of ring worm infections, and as timber, the wood is white to yellow, often streaked with brown, pink, red or green; it is hard and heavy but usually not hard enough to make anything but small articles. The ***chief use of this tree is magical***. The plant is used as traditional medicine. A decoction of the root is taken as a blood purifier, to treat pneumonia, gonorrhea, rheumatism and stomach-ache and as a gargle. The leaves are applied as liniment to simple sprains. Leaf decoction mixed with the bark of *Erythrina abyssinica* is used to alleviate stomach pain. It is also used in the treatment of skin complaints and respiratory infections. In Southern Africa, the chief use of the tree is for magic; it is widely used as a protective charm toward off lightning and evil influences from homes and crops, and to bring luck in hunting. The South Sotho name 'Mofifi' means 'darkness', and in Lesotho they say "darkness overcomes witchcraft" [7, 8, 10].

In Ethiopia, *Gesho* is used in the preparation of domestic beverages such as *Tella* and *Tej* [12]. The leaves and stems of *Gesho* are indispensable ingredients in the making of these traditional fermented beverages. It has been reported that the plant regulates the microflora responsible for the fermentation process [12, 13]. It is believed that *Gesho* can serve as a commercial hopping agent in the brewery industries. Hops add bitterness *via* alpha acids

being isomerised into more stable and soluble iso-alpha acids assist in the production of tannins that combine with unwanted proteins, add to beer stability due to their antibacterial properties, and impart characteristic aroma to beer through their essential oils [14, 15]. It has been speculated that the role of *Gesho* in *Tella* should be similar to that of hops in beer.

It plays a major role to suppress certain bacteria during the fermentation process. *Gesho* is certainly the main agent that imparts the desirable bitter taste to *Tella*. Its contribution to the typical aroma of these beverages is due to Geshoidin; a previously unknown naphthalenic compound β-*sorigenin-8-O*-β-*D-glucoside.* It is interesting to know that unlike hops, *Gesho* does not contain essential oils. A number of compounds have been isolated from the leaves and stems of *Gesho* [16, 17, and 18]. Previously unknown naphthalenic compound β-*sorigenin-8-O*-β-*D-glucoside* (Geshoidin), (Figure 3 below) was extracted, isolated and purified from the leaves and was characterized by spectroscopic analysis. The name *Geshoidin* was proposed for this novel glucoside. The discovery of *Geshoidin* is very significant in the study of the chemistry of *Gesho*, because the characteristic bitter taste of this plant was ascribed by organoleptic evaluation to Geshoidin, one of the major constituents of the plant [12, 16].

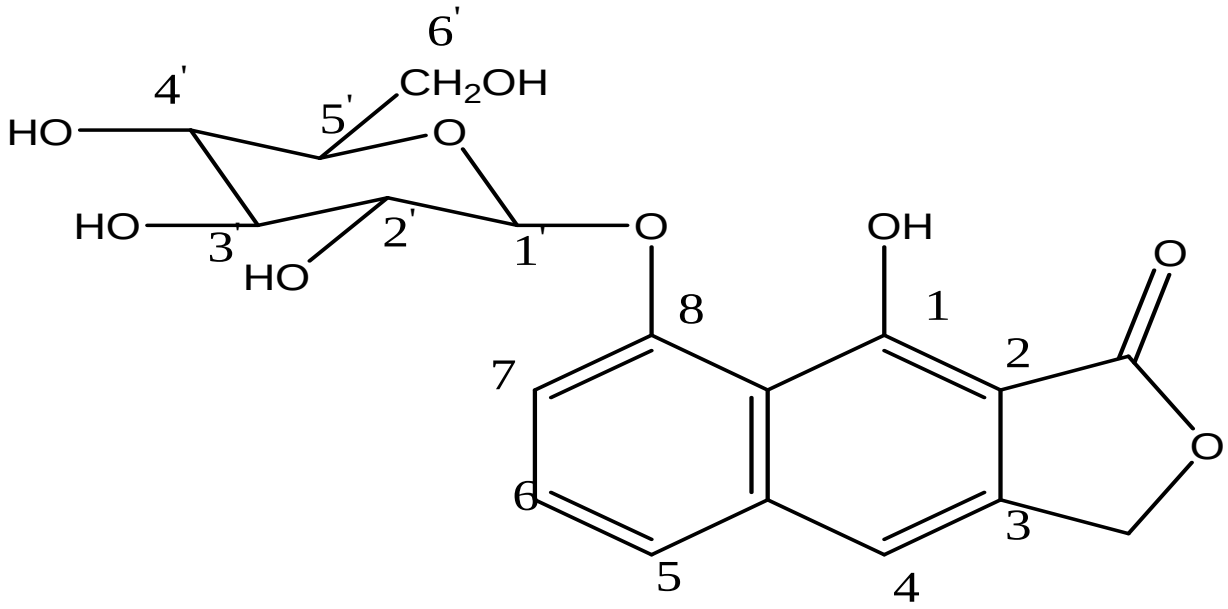


Figure 3: Structure of *Geshoidin* (β-*sorigenin-8-O*-β-*D-glucoside*).

1.4.3. Structure determination of Geshoidin.

The structure determination of organic compounds is determined by the spectroscopic techniques, mainly nuclear magnetic resonance spectroscopy, NMR (both ^{1}H and ^{13}C NMR) – which determines the map of carbon-hydrogen framework, and infrared (IR) spectroscopy – which determines the molecular functional groups. The other equally important spectroscopic techniques are mass spectrometry (MS) which determines the molecular size and the formula and ultraviolet visible spectroscopy (UV-Vis) which determines the presence of conjugated π electron system [17]. Geshoidin structure too, was determined mainly from ^{1}H and ^{13}C NMR and IR spectroscopic techniques [13].

The information obtainable from the ^{1}H NMR is that, it shows the types and the number of protons of the molecule (which differ in the chemical environment) as well as the number of neighbouring protons within that molecule. Therefore, ^{1}H NMR spectrum of Geshoidin revealed the presence of four aromatic protons at positions 4, 5, 6 and 7 with appropriate chemical shifts.

^{1}H NMR (300 MHz, DMSO-d_6): δ 3.30 – 3.75 (6H, glucoside), 5.06 (d, H-1', glucoside, J =7.8), 5.26 (d, lactone CH_2), 7.27 (s, H-4), 7.49 (d, H-5, J = 7.2), 7.45 (t, H-6, J = 7.3), 7.33 (d, H-7, J = 7.2), 10.45 (s, OH-1) [13], where J is the coupling constant. The coupling constant of 7.8 for doublet H-1' from the glucoside moiety implies that H-1' and H-2' are in axial – axial positions since the axial – axial coupling constant (Jaa is around 8) and therefore the sugar moiety is a β-D-glucoside.

The information obtainable from ^{13}C NMR on the other hand, is the number of carbons present in the molecule (which differ in chemical environment). Therefore, ^{13}C NMR spectrum of Geshoidin revealed 12 carbons on sorigenin ring system with the corresponding chemical shifts (table 1). The DEPT ^{13}C NMR (distortionless enhancement by polarization transfer) indicated the presence of two CH_2, (one from lactone CH_2 and one from C-6', glucoside), nine CH and seven quaternary carbons and the absence of CH_3 group, consistent with the molecular formula $C_{18}H_{18}O_9$ obtained from mass spectrometry [13].

Table 1: ^{13}C NMR spectra of Geshoidin measured in DMSO-d_6

C #	1	2	3	4	4a	5	6	7	8
δ	155.3	114.3	139.3	111.0	142.9	123.0	129.5	111.0	155.8

C #	8a	Lactone CH_2	Lactone CO	1’	2’	3’	4’	5’	6’
δ	105.8	67.9	168.8	102.9	73.4	77.8	69.9	72.3	60.8

The overall analysis of IR, ^{1}H NMR and ^{13}C NMR properties resulted into naphthalenic lactone glycoside.

1.5. Antioxidant properties of Geshoidin.

The evidence suggests that the reactive oxygen species and their promoted oxidative damage are involved in a large number of pathologies as well as in the aging process [18]. Under normal metabolism, the active oxygen radicals produced by cells and scavenged by cells themselves are in stable equilibrium. If they are maladjusted, many kinds of diseases occur due to overabundance of free radicals in *vivo*. Therefore, how to prevent radicals and active oxygen from the harm to organism tissue has become a very interesting area of investigation for many researchers. Polyphenols are characterised by the presence of one or more hydroxylated benzene rings and are known for their radical scavenging ability [18, 19]. Owing to the phenolic nature of *Geshoidin*, its ability of scavenging active oxygen radicals or its chemical antioxidant activity has been studied before by the previous researchers [18].

1.6. Extraction, isolation and purification of Geshoidin from Rhamnus Prinoides.

Extraction: Separation between two phases for example, when the sample (in this case, Gesho) is initially present in one of the phases (solid phase), the separation is known as an ***extraction*****.** In a simple extraction the sample is extracted one or more times with portions of the second phase (usually the solvent, in this case methanol). Simple extractions are particularly useful for separations in which only one component has a favourable distribution ratio [19]. Several important separation techniques are based on simple extractions, including liquid–liquid, liquid–solid, solid–liquid, and gas–solid extractions, (in this case, solid-liquid extractions) [19, 20].

Chromatography is a physical method of separation (isolation) in which the components (extracts) to be separated are distributed between two phases, one of which is stationary

(stationary phase) while the other (the mobile phase) moves in a definite direction. The mobile phase moves through the chromatography column (the stationary phase) where the sample interacts with the stationary phase and is separated. Preparative chromatography is used to purify sufficient quantities of a substance for further use, rather than analysis as opposed to analytical chromatography.

1.6.1. Two methods of chromatography techniques that were used for the purification of Geshoidin are as follows.

1. ***Column chromatography*** is a separation technique in which the stationary bed (in this case, oxalic acid impregnated silica gel) is packed within a tube (glass column). The particles of the solid stationary phase or the support coated with a liquid stationary phase may fill the whole inside volume of the tube (packed column) or be concentrated on or along the inside tube wall leaving an open, unrestricted path for the mobile phase in the middle part of the tube (open tubular column). In this technique, the mobile phase moves under the influence of gravitational pull [20, 21]. The efficiency of the column for better separation is ascribed to the chromatographic separation theories, namely - the plate theory and the kinetic theory formulated by Martin and Synge [19, 20].

The plate theory says that the chromatographic column is treated as though it consists of the discrete sections of the equilibrium zones or plates where partitioning between the mobile and the stationary phases occurs. Therefore, the column efficiency is described by the number of the theoretical plates (N), the height of the theoretical plate (H) and the length of the column packing (L) defined by equation (1) below:

$$N = \frac{L}{H} \quad \text{..... (1)}$$

Equation (1) shows that the longer the column packing the better the separation, although at the expense of the longer analysis time, the more the number of the theoretical plates for partition or adsorption equilibration, the better the separation and the shorter the theoretical plate height, the better the separation, or more generally, the better the column efficiency.

The kinetic theory on the other hand says that the resolution (R) equation is given by:

$$R_G = \frac{1}{4}\sqrt{N}\left[\frac{\alpha-1}{\alpha}\right]\left\{\frac{K'}{K'+1}\right\} \quad \text{..... (2)}$$

$$T_{R,G} = \frac{16R^2H}{u}\left(\frac{\alpha}{\alpha-1}\right)^2\frac{\{1+k'\}^3}{\{K'\}^2} \quad \text{..... (3)}$$

Where; α is the selectivity factor and K' is the capacity factor for the column packing, $T_{R,G}$ is the retention time based on Geshoidin and u is the mobile phase velocity. Equation (2) still illustrates that the resolution which is directly related to the separation, improves with the increase in the number of the theoretical plates, and hence the overall column efficiency as in equation (1). Equation (3) demonstrates that the time the Geshoidin is retained by the stationary phase increases with the increase in resolution and the decrease of the mobile phase velocity.

2. ***Thin layer chromatography (TLC)*** is a widely employed laboratory technique and is similar to paper chromatography. However, instead of using a stationary phase of paper, it involves a stationary phase of a thin layer of adsorbent like silica gel, alumina, or cellulose on a flat, inert substrate. Compared to paper, it has the advantage of faster runs, better separations, and the choice between different adsorbents. For even better resolution and to allow for quantification, high-performance thin layer chromatography (TLC) can be used. An eluent (solvent) soaks through the adsorbent material carrying the components of the mixture at different rates upwards [21].

1.7.0. Charactarisation by electrochemical means.

1.7.1. General overview of Electrochemical Methods.

Some electrochemical cells employ only two electrodes while others employ the three electrode systems. In potentiometric measurements (zero-static current conditions such as measurement of pH), there is no current through the cell, and the two electrodes are sufficient. However, in a cyclic voltammetry experiment, an external potential is applied to the cell, and the current response is measured. Precise control of the external applied potential is required, but this is generally not possible with a two electrode system, due to the potential

drop across the cell due to the solution resistance (potential drop (E) = current (i) x solution resistance (R)) and the polarization of the counter electrode that is required to complete the current measuring circuit. Better potential control is achieved using a three electrode system with a potentiostat which controls the potential between the working electrode (WE) and the reference electrode (RE with a constant potential), while the current passes between the WE and the third electrode called the auxiliary electrode , AE [22, 23].

The existence of the current response with the applied potential in voltammetry arises from transfer of electrons between the energy levels of the working electrode and the molecular energy levels of the system under study. This current is often referred to as the *faradaic* current. Transfer of electrons from filled electrode orbitals to vacant molecular orbitals is referred to as reduction (the gain of electrons), whereas transfer of electrons from filled molecular orbitals to vacant electrode orbitals is referred to as oxidation (loss of electrons).

Whether oxidation or reduction can occur depends upon the relative energies of the Fermi level of the electrode (i.e. the energy of the highest occupied electrode orbital) and the frontier molecular orbitals; for example, reduction can occur if the Fermi level is higher than the lowest unoccupied molecular orbital (LUMO), whereas oxidation requires that the Fermi level be lower than the highest occupied molecular orbital (HOMO) [23, 24]. The Fermi level is determined by the potential applied to the electrode; that is, varying the applied potential changes the oxidizing/reducing ability of the electrode. For example, more negative potentials increase the reducing ability of the electrode.
In contrast, the energies of the molecular frontier orbitals are determined by the molecular structure and can be considered to be constant. Therefore, a common approach in voltammetry experiments is to vary the applied potential, and to record the potential at which a current response is detected; that is, the energy at which oxidation or reduction occurs. The *redox potential* is a measure of this energy [24].

Although all molecules do have frontier orbitals, in practice these are not always accessible in a voltammetry experiment. Molecules for which a redox potential can be measured are referred to as electrochemically active. Examples of electrochemically active molecules include organic molecules with extended pi-systems (e.g., aromatic molecules) and transition metal complexes. It should also be noted that some systems have the ability to undergo more than one oxidation or reduction, and hence have more than one redox potential [24, 25].

1.7.2. The effect of stirring the solution and deoxygenating.

Stirring the solution has a significant effect on the current response, since it affects the rate at which electroactive molecules are brought from the bulk solution to the electrode surface (this process is referred to as *mass transport*). In many voltammetry experiments, there is no stirring, and the only form of mass transport is diffusion (this gives rise to the tailed peak shape observed in cyclic voltammetry). These are referred to as *stationary solution* techniques. In other experiments, the solution is stirred; either by a stir bar or a rotating electrode (the latter is preferable, due to the more precise control of the rate of rotation). These are referred to as *hydrodynamic* techniques [26].

Oxygen is electroactive, and can be reduced quite easily. Therefore, it must be removed from the solution if the system under study is reducible. Oxygen is typically removed by bubbling an inert gas (e.g., nitrogen or argon) through the solution for about 10 minutes. If a stationary solution experiment is to be performed, it is important that the stirring is stopped and the solution is allowed to become quiescent before the experiment is started (although a blanketing layer of inert gas over the solution can be maintained during the experiment [27].

1.7.3. Cyclic voltammetry (CV) in general.

Cyclic voltammetry (CV) is one of the most commonly used electrochemical techniques, for acquiring qualitative information about electrochemical reactions, and is based on a linear potential waveform; that is, the potential is changed as a linear function of time. The rate of change of potential with time is referred to as the *scan rate*. The simplest technique that uses this waveform is Linear Sweep Voltammetry (LSV). The potential range is scanned starting at the Initial potential and ending at the Final potential. CV is an extension of LSV in that the direction of the potential scan is reversed at the end of the first scan (the first Switching Potential), and the potential range is scanned again in the reverse direction. The experiment can be stopped at the Final Potential, or the potential can be scanned past this potential to the second Switching Potential, where the direction of the potential scan is again reversed. The potential can be cycled between the two Switching Potentials for several cycles before the experiment is ended at the Final Potential [24, 26 27]. Therefore, depending on the information sought, the single or multiple cycles can be used.

The power of CV results from its ability to rapidly render considerable information on the thermodynamics of the redox processes, on the kinetics of heterogeneous electron- transfer reactions, and the coupled chemical reactions or adsorption processes [27]. It often offers a rapid location of redox potentials of the electroactive species and convenient evaluation of the effect of media upon the redox processes. CV consists of scanning linearly the potential of the stationary working electrode (in an unstirred solution) using a triangular potential waveform usually referred to as the voltage sweep program (figure 4). The potentiostat measures the current resulting from the applied potential. The resulting plot of the current versus potential is termed a *cyclic voltammogram,* illustrated by a reversible redox couple during a single potential cycle (figure 5).

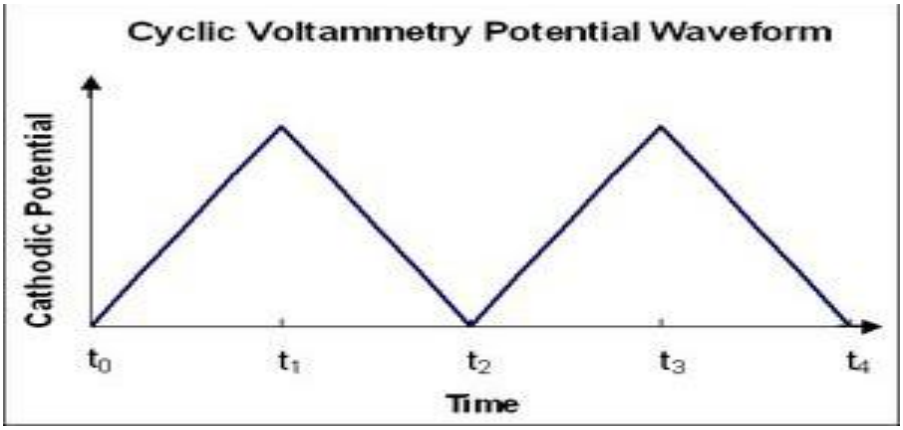


Figure 4: typical voltage sweep program (potential-time excitation signal) in CV experiment. From $t_o - t_1$ is the forward scan while from t_1- t_2 is the reverse scan and from $t_o - t_2$ is the full cycle. The switching potential is at t_2 [24].

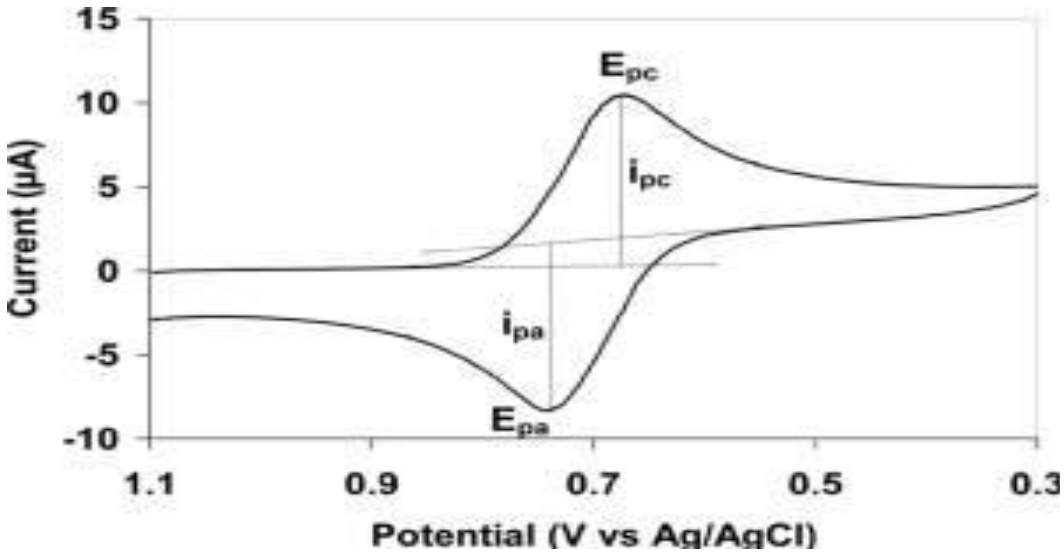


Figure 5: typical cyclic voltammogram for a reversible O + ne^- = R redox process. E_{pc} and E_{pa} are cathodic and anodic peak potentials respectively. i_{pc} and i_{pa} are the cathodic and anodic peak currents respectively.

The peak current for a reversible process is given by the Randles-Sevick equation at 298K.

$$\boldsymbol{i_p = (2.69 \times 10^5) n^{3/2} A D^{1/2} C \nu^{1/2}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots .. \textbf{(1)}$$

Where ***A*** is the electrode surface area (cm^2) obtained using geometric measurement, or more accurately, by chronocolometry, ***D*** is the diffusion coefficient ($cm^2\ s^{-1}$), ***C*** is the concentration of the electroactive species in the bulk solution (mol cm^{-3}) and **ν** is the scan rate (Vs^{-1}). Therefore, $\boldsymbol{i_p}$ is proportional to ***C*** and proportional to $\nu^{1/2}$. If ***A*** is known, then ***D*** can be calculated from the slopes of the linear plots of $\boldsymbol{i_p}$ versus ***C*** and $\boldsymbol{i_p}$ versus $\nu^{1/2}$ [29].

The ratio of the reverse to forward peak currents, i_{pr} / i_{pf}, is unity for the reversible couple. The position of the peaks on the potential axis (E_p) is related to the formal potential (E^0) of the redox process. The formal potential of the reversible couple is centered between E_{pc} and E_{pa}:

$$\mathbf{E^0 = (E_{pa} + E_{pc}) / 2} \ \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \textbf{(4)}$$

The separation between the peak potentials for the reversible couple is given by:

$$\mathbf{\Delta E_p = |E_{pa} - E_{pc}| = 0.059 / n\ V} \ \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \textbf{(5)}$$

Thus, the peak separation can be used to determine the number of electrons transferred, and as the criterion for a Nernstian behavior [29].

Cyclic voltammetry can also be extended over irreversible processes and or quasi-reversible systems such as in the case of electrochemical responses of Geshoidin which is chemically irreversible [17]. For irreversible processes (those with sluggish electron exchange), the individual peaks are characterized by a shift of the peak potential with the scan rate.

$$\mathbf{E_p = E^0 - RT / \alpha n_a F\ [0.78 - \ln (K^0/D^{1/2}) + \ln (\alpha n_a/RT)^{1/2}]} \ \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \textbf{(6)}$$

Where α is the transfer-coefficient and n_a is the number of electrons involved in the charge-transfer process. Therefore, E_p occurs at the potentials higher than E^0, with the over potential related to K^0 and α. Independent of the value of K^0, such peak displacement can be counterbalanced by an appropriate change of the scan rate (V). The peak potential and the half-peak potential (at 298K) will differ by 48/αn mV. Hence, the voltammogram becomes more drawn-out as αn decrease [27, 28, 29].

1.7.4. Modification of the glassy carbon working electrode (GC-WE).

Electrochemical behaviour of Geshoidin can be determined on the bare glassy carbon WE, but quantitation can still be enhanced using the modified glassy carbon WE in some cases. The WE can be modified with carbon nanotubes in nafion or any highly conducting polymers such as poly (3,4-ehylenedioxythiophene) or PEDOT, either by electrochemical means (e.g. cyclic voltammetry) or putting a drop on the surface of the electrode and then letting it to dry. Multi-wall carbon nanotubes (CNT) have attracted much attention in nano technology as the chemical modifiers because they have the ability to promote electron transfer reactions when used as the electrodes materials, moreover CNT are very stable and insoluble in most solvents, which restrained its application in electroanalysis [31], the only disadvantage being that of electrode fouling with time. On the other hand, PEDOT as conducting polymers are widely employed as coatings conferring the electrode systems antifouling properties and possibly activating the electrocatalytic redox processes [30, 31, 32].

1.8. Aim of the project.

The aim of this project was to extract and isolate Geshoidin from the leaves of *Rhamnus Prinoides* and then, study its electrochemical properties.

2. EXPERIMENTAL.

2.1 Apparatus.

1L Separatory funnel, 250mL Erlenmeyer flasks, Rotary evaporator, 500mL Florentine flasks for rotary evaporator, 250mL glass column chromatography, analytical thin layer chromatography (TLC): silica gel plates and melting point apparatus. All of the above were needed for extraction and isolation procedures. For electrochemical procedures, a model BAS 100B electrochemical analyser (Bioanalytical Systems) was used for cyclic, and differential pulse voltammetry measurements, with a three-electrode system consisting of a glassy carbon disk working electrode (BAS MF-2012) with an active surface area of 0.06 cm^2, an Ag/AgCl (3 M NaCl) reference electrode (BAS MF-2052) and a platinum wire auxiliary electrode (BAS MW-1032). Before each experiment the glassy carbon electrode was polished manually with alumina (f: 0.01 μm) on a micro-cloth pad and rinsed with distilled and de-ionized water. All potentials were reported with respect to Ag/AgCl (3 M NaCl) reference electrode.

2.2 Reagents.

Plant material (250g): Rhamnus Prinoides L' Herit leaves were provided by Professor Alemayehu C. G. (supervisor) for extraction and isolation procedures. Other chemical reagents were obtained from the Department of Chemistry and Chemical Technology, namely: methanol (MeOH 100%), oxalic acid, and silica gel, chloroform ($CHCl_3$) and ethylacetate (EtOAc).

For electrochemical procedures, the purified Geshoidin compound, Citric acid and di-sodium hydrogen phosphate from Saarchem (South Africa), ascorbic acid, citric acid, glucose and sodium perchlorate from Riedel-de Haen (Germany), and sodium hydroxide from ACE (South Africa) were used as received. Distilled, de-ionized water was used throughout. A 1 x 10^{-2} mol dm^{-3} aqueous standard stock solution of *Geshoidin* was prepared and stored in the dark to protect it from light. The required concentration was then prepared from the stock standard solution daily. Citric acid/di-sodium hydrogen phosphate buffer system in the pH range 2 - 11 were prepared from mixture of 0.1 mol dm^{-3} citric acid and 0.2 mol dm^{-3} di-

sodium hydrogen phosphate aqueous solutions. The pH of the solutions for higher values was adjusted by adding drops of 1 mol dm^{-3} sodium hydroxide solution

2.3. Procedure.

Procedure A: (Extraction and isolation)

2.3.1. Extraction of Geshoidin from the leaves of Gesho.

250g of the powdered leaves of Gesho were measured and contained in 1L separatory funnel with ~ 500mL MeOH for extraction for 1 week. A 344.2g Florentine flask was used to collect the extracts from the separatory funnel and evaporated them with rotary evaporator to recover MeOH solvent and led to 78.2g of the residue. The recovered MeOH was fed back into the separatory funnel to ensure maximum extraction and also as to save the fresh solvent.

2.3.2. Preparation of oxalic acid impregnated silica gel.

5% of oxalic acid solution was prepared by dissolving 5g of solid oxalic acid in 95g (95mL) of distilled water. 100g of silica gel was weighed and soaked with 5% oxalic acid solution to prepare an impregnated oxalic acid / silica gel. The impregnated oxalic acid silica gel was put into the oven for 24 hours to remove the adsorbed water molecules.

2.3.3. Isolation of Geshoidin from the extracts.

10g of the extracted residue was mixed with 15g of dry oxalic acid-impregnated silica gel and transferred 1:10 mixture of the extract to oxalic acid – impregnated silica gel already packed into a 250mL glass column. After adding the extract into the column, the chloroform solution was added over the packed sampled column with no air bubbles so as to maximize the efficiency of the column. 10 fractions (eluates) were collected from the chromatographic column with $CHCl_3$ solvent as the eluent and run their preparative TLC chromatography. Fractions 1, 2-3 were similar based on coloured compounds and were added together. Fractions 4, 5, 6-7 had the same colour but different from fractions 1, 2-3 and they were also added together. Fractions 8-9 were also collected, exhibiting the same coloured compounds but different from other fractions and were also added together, fraction 10 was collected very dilute in terms of the coloured compounds and the solvent / eluent was recovered with the rotary evaporator from these eluates or fractions.

Fractions 11-12 were collected with the EtOAc eluent and resulted into almost similar coloured compounds but different from fractions 1-10. The polarity of the eluents / solvents was adjusted by combining 2:1 (EtOAc: MeOH) solvents and eluted fractions 13, 14-15 and these fractions were believed to have contained Geshoidin since it elutes with moderately polar solvents.

1.3.4. Confirming the purity of Geshoidin with the melting point determination.

The sample of an isolated Geshoidin was subjected to melting point apparatus with the melting point tube and recorded its melting point.

2.3.5. Confirming the purity of Geshoidin with the preparative TLC chromatography.

2:1 EtOAc: MeOH solvent system (mobile phase) was prepared and saturated the TLC tank for ~ 2minutes. The Geshoidin eluates (fraction 13, 14-15) and the standard pure Geshoidin were spotted on the TLC plates and put in the saturated TLC tank for TLC separation. Geshoidin was also purified by recrystallizing it in methanol.

Procedure B: (Characterisation by electrochemical)

2.4. Electrochemical behaviour of Geshoidin using a BAS 100B electrochemical analyzer with a three electrode system.

Cyclic voltammetric measurements were run from -1.200 to + 1.200 V and back at a glassy carbon electrode with a scan rate of 100 mV s^{-1}. The scan rate was varied from 0.005 to 0.2 V s^{-1} to study the dependence of peak current and peak potential on the scan rate.

Controlled potential electrolysis of *Geshoidin* was carried out at a glassy carbon electrode of large surface area (0.79 cm^2) in citric acid/di-sodium hydrogen phosphate buffer for three concentrations of *Geshoidin* (C = 1 x 10^{-5}, 2 x 10^{-5}, 4 x 10^{-5} mol dm^{-3}). Solutions were stirred during electrolysis using a magnetic stirring bar. The electrolysis was terminated when the

electrolytic current decreases to the residual current value measured in the supporting electrolyte prior to the addition of the analyte.

20 mL supporting electrolyte was placed in the electrochemical cell and the required volume of standard *Geshoidin* solution was spiked into the cell by micro-pipette. The same procedure was followed for sample analysis. The solution was deaerated with pure nitrogen (99.999%, Air Products SA). 200 μL of the extract was spiked into the electrochemical cell that contained 10 mL of buffer solution and linear scan voltammograms were recorded. The standard addition method was applied adding successive aliquots of 20 μL of 1 x 10^{-2} mol dm^{-3} *Geshoidin* standard solution to the electrochemical cell. Linear scan voltammograms were recorded by scanning anodically from 0.000 to 0.800 V at scan rate of 20 mV s^{-1}. The peak current of the oxidation wave at about 0.550 V was measured. After each experimental run, the solution was stirred for 10 s prior to the next measurement.

3. RESULTS AND DISCUSSIONS.

3.1. Separation by extraction.

Methanol extraction of the leaves and the removal of the solvent (MeOH) by rotary evaporator resulted in 78.0g of the residue implying that the extraction was efficient since the expected mass of the residue was 13.9g relative to the procedure described by [13] and simply because the extraction was performed for a week. The choice of the solvent (MeOH) which is a very polar organic solvent was due to the fact that the compound of interest, Geshoidin (naphthalenic lactone glycoside) is polar in nature and therefore, the organic polar solvent was of paramount importance since the like dissolves the like by the rule of thumb in chemistry. The recovered MeOH was fed back into the extraction system to ensure maximum extraction.

3.2. Preparation of chromatographic column.

The column packing was prepared by impregnating silica gel with oxalic acid aqueous solution and tried in the oven for 24 hours, making sure that all adhered water molecules have been driven away so that the polarity of the packed column was enhanced a little bit, and not too much. The purpose of preparing the stationary phase of silica gel with the aqueous solution of oxalic acid was to enhance the polarity of the stationary phase so that, since the polar naphthalenic glucoside will spend a little bit more time in the stationary phase, so that the $T_{R,G}$ (equation 3) is larger and hence, the maximum partitioning of Geshoidin between the stationary phase and the mobile phase is accomplished. Another purpose of impregnating the silica gel with oxalic acid was to acidify the column packing since the naphthalenic lactonic glycoside is naturally acidic. Therefore, acidifying the column had an advantage of improving the efficiency of the column separation since the compound to be isolated was acidic too. After the column was packed, enough volume of $CHCl_3$ was added on top to make sure that the column packing adhered itself strong enough onto the surface of the glass column without any air bubbles within the column packing so as not to disturb the column packing efficiency.

3.3. Separation by column chromatography i.e. isolation.

The portion of the extract (residue) was then subjected to the oxalic acid-impregnated silica gel packed column chromatography for isolation of Geshoidin from other compounds in the leaves of Gesho. The less polar compounds such as chrysophanol, physcion and naphthalenic phenol, musizin from the leaves of Rhamnus Prinoides [13] were isolated first using

chloroform ($CHCl_3$) as the mobile phase since it is a less polar solvent and therefore, it was assumed to elute less polar compounds of the extracts.

Ten fractions (eluates) were collected from the column using the same mobile phase, $CHCl_3$, which were assumed to be other compounds of Rhamnus Prinoides of no interest in this project. These fractions were combined together and the attempt to recover $CHCl_3$ was made but failed to recover it simply because $CHCl_3$ is about half as dense as water and the rotary evaporator's temperature was set very low. Therefore, the resulting solution was used to clean the laboratory glass ware. Since the tenth eluate was collected very dilute in terms the coloured compounds, then, it implied that $CHCl_3$ was no longer eluting anything in terms of the coloured compounds and therefore, the mobile phase was changed to ethylacetate (EtOAc) to obtain fraction 11-12.

The elution time when $CHCl_3$ was used as the mobile phase was pretty satisfactory because of high density of $CHCl_3$, enabling it to elute faster due the gravitational pull. The faster the elution time, the better the column efficiency and this is what is required in chromatographic separation [19, 20]. The elution time when using EtOAc as the mobile phase was very long, since EtOAc is less dense and that is not favoured in chromatographic separation, since the analysis time is longer.

The polarity of the mobile phase was adjusted by combining a 2:1 ratio of EtOAc: MeOH to elute fractions 13, 14-15. These fractions were believed to have contained Geshoidin since it practically elutes with moderately polar organic solvents. Fractions 13, 14-15 were found to contain the trace amounts of the brownish tinge precipitate which was believed to be the novel glucoside, Geshoidin. Its mass after trying for a very long time (one week) was found to be 36.8mg out of 9.95g of the extract.

3.4. Identity of the isolated Geshoidin.

Geshoidin was identified by the very simple but precise methods, namely; melting point determination and the preparative TLC chromatography. The melting point (mp.) of the isolated Geshoidin was found to be 157 – 161 ^{0}C, while the theoretical melting point is 160 – 162 ^{0}C. The observation follows that the isolated Geshoidin was slightly impure.

3.5. Electrochemical behaviour of Geshoidin.

3.5.1. Cyclic voltammetry of Geshoidin

The electrochemical responses of *Geshoidin* were investigated using cyclic voltammetry. Figure 6 shows the cyclic voltammogram (CV) of 5 x 10^{-5} mol dm^{-3} *Geshoidin* at a glassy carbon electrode in pH 7.0 citric acid/di-sodium hydrogen phosphate buffer. The CV shows the current potential profile of *Geshoidin* after subtracting the CV of the base electrolyte. During the first positive potential scan, an irreversible oxidation peak appeared at a potential of 0.522 V followed by a hump at 0.675 V. During the reverse negative potential scan, an irreversible reduction peak was observed at -0.690 V. The reduction peak was not seen when the starting potential of the CV was set first at +1.200 V and scanned in the negative direction up to a switching potential of -1.200 V. This implies that the reduction peak is attributed to the reduction of the oxidised species of *Geshoidin*. When repetitive cycles were run at low scan rates, no change was observed in the shape and height of both the oxidation and reduction peaks indicating the absence of electrode surface fouling due to strong adsorption or polymerisation of the electro reactive species. However, the hump that followed the oxidation peak and observed at 0.675 V during the first scan of the voltammogram is an indication of the existence of weak adsorption of the oxidation product [17]. The peak disappeared when the experiment was run for repetitive cycles or at different scan rate

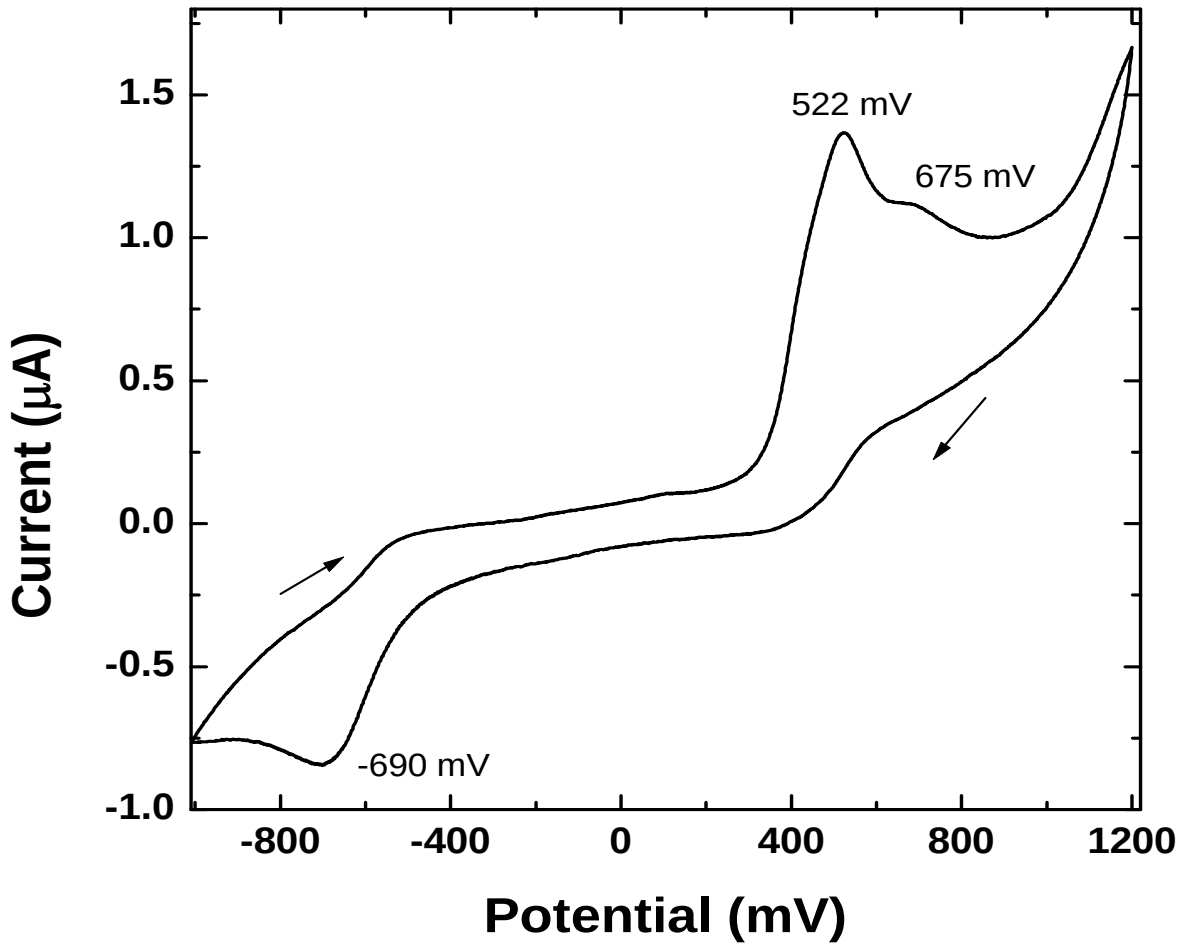


Figure 6: Cyclic voltammogram of 5 x 10^{-5} mol dm^{-3} *Geshoidin* at a glassy carbon electrode in a mixture of 0.1 mol dm^{-3} citric acid and 0.2 mol dm^{-3} di-sodium hydrogen phosphate buffers (pH 7) after subtraction of base electrolyte's voltammogram at a scan rate of 60 mV s^{-1}.The oxidation and reduction of *Geshoidin* at glassy carbon electrode gave rise to chemically irreversible processes over the scan rate range of 5 mV s^{-1} to 5 V s^{-1}. Figure 7 shows the cyclic voltammograms for the oxidation of 5 x 10^{-5} mol dm^{-3} *Geshoidin* solution at different scan rates. The peak potential for the process became more positive as the scan rate increased while the peak currents were proportional to the square root of the scan rate, for the scan rate up to 200 mV s^{-1} (Figure 7) as expected when the mass transport process is diffusion controlled [22, 23]. At scan rates greater than 200 mV s^{-1}, the oxidation process lost the characteristic diffusion controlled peak shape and became broad and sigmoidal implying that surface based process becomes dominant at high scan rates [17, 18].

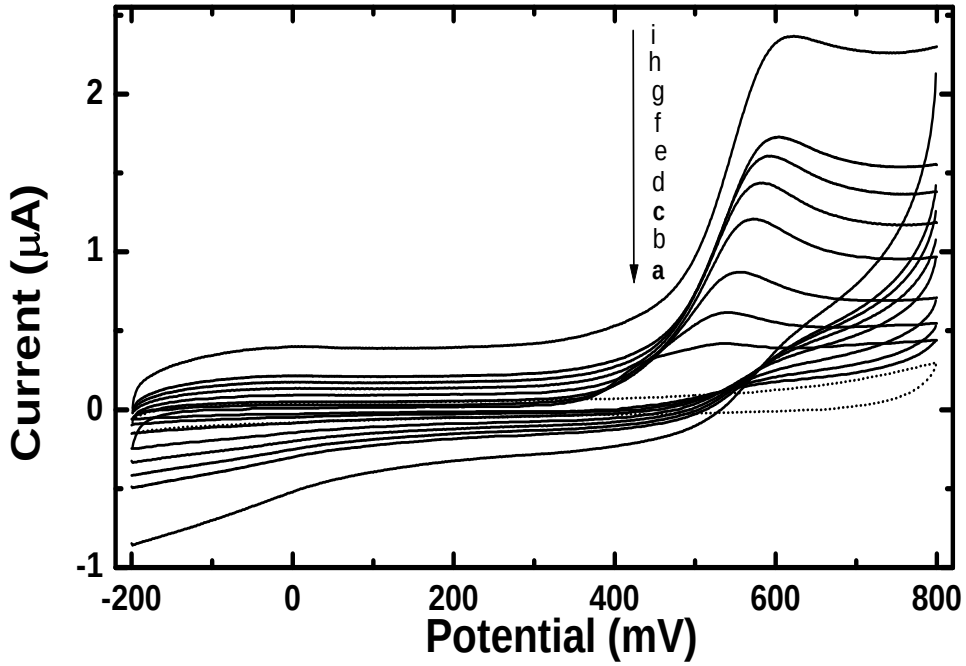


Figure 7: Cyclic voltammograms of 5 x 10^{-5} mol dm^{-3} *Geshoidin* at a glassy carbon electrode in a mixture of 0.1 mol dm^{-3} citric acid and 0.2 mol dm^{-3} di-sodium hydrogen phosphate buffer (pH 7) at different scan rates: (a) base electrolyte; (b) 5; (c) 10; (d) 20; (e) 40; (f) 60; (g) 80; (h) 100 and (i) 200 mV s^{-1}.

The effect of the potential scan rate, ν on the oxidation peak current of *Geshoidin* was studied and the oxidation peak current was proportional to the square root of scan rate, $\nu^{1/2}$ in the entire pH range studied as predicted for a diffusion controlled regime. Plot of the peak current as a function of the square root of the scan rate for Fig. 7 is described by the following equation.

$$i_p/\mu A = 0.13/\mu A + 4.32\nu^{1/2} \qquad r^2 = 0.996 \qquad \text{(for n=8)}\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (7)$$

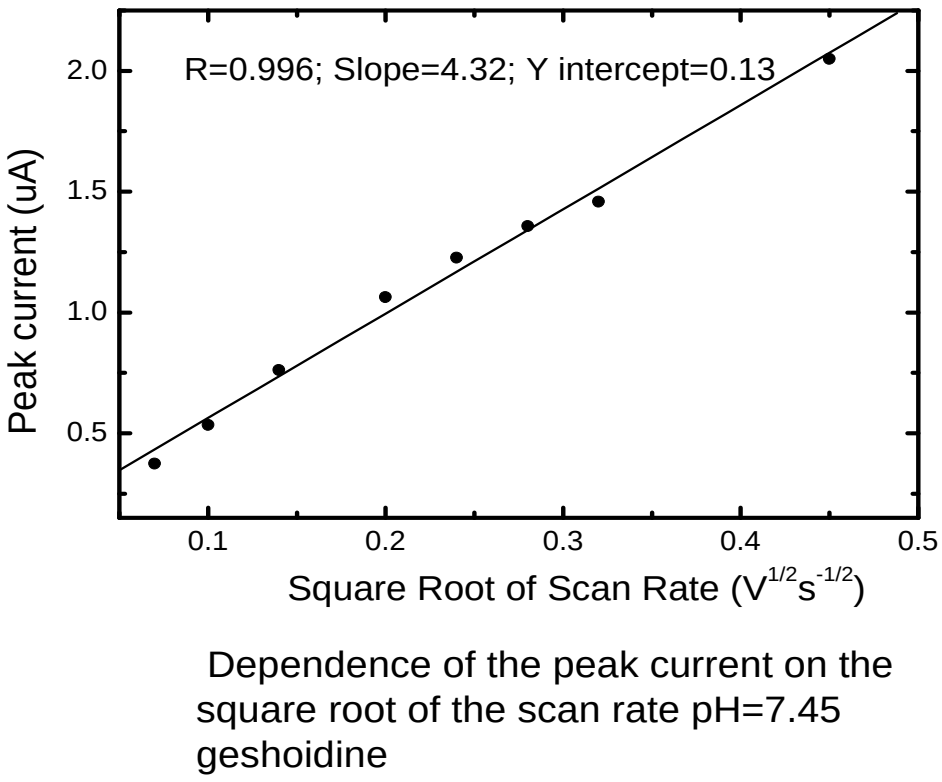
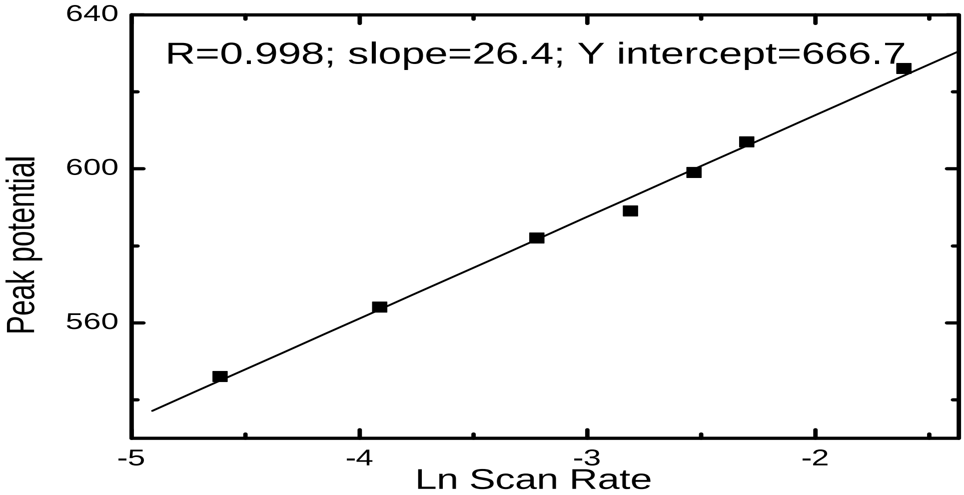


Figure 8: Dependence of the peak current on the square root of the scan rate (pH 7.45) of Geshoidin.

The dependence of the oxidation peak potential of *Geshoidin* on the logarithm of the potential scan rate for Fig. 9 was evaluated and the peak potential was directly proportional to the logarithm of the scan rate and the linear plot is expressed as follows.

$E_p/V = 0.667 + 0.01284\ln\nu$ $r^2 = 0.998$ (for n=8) …….. (8)



Dependence of peak potential on the logarithm of scan rate for 0.05 mM Geshoidine.

Figure 9: Dependence of peak potential on the logarithm of scan rate for 0.05 mM Geshoidin.

Constant potential electrolysis of *Geshoidin* was carried out at 0.700 V for three concentrations of *Geshoidin*, (C = 1 x 10^{-5}, 2 x 10^{-5}, 4 x 10^{-5} mol dm^{-3}) to determine the number of electrons transferred in the process. From the electrolysis results, the average number of electrons n transferred per molecule was found to be 2.1 ± 0.2. For a totally irreversible oxidation reaction the peak current at 25 ^{o}C is given by:

$$i = (2.99 \times 10^{5}) n[(1-\alpha)n_{\alpha}]^{1/2} A C_{b} D^{1/2} \nu^{1/2} \quad \text{(9)}$$

Where A in cm^2, D in $cm^2\ s^{-1}$, C_b in mol cm^{-3}, ν in V s^{-1} and n_{α} is the number of electrons transferred up to, and including the rate determining step [33]. The peak potential is related to the scan rate ν with the following relation.

$$E_p = K + [RT/2(1-\alpha)\, n_{\alpha}F] \ln\nu \quad \text{(10)}$$

Where $K = E^{o} + [RT/(1-\alpha)\, n_{\alpha}F][0.78 + (1/2)\ln [(1-\alpha)\, n_{\alpha}F\, D/k^{o2}RT]$

From equation (10) and t = 25 ^{o}C, the value of $(1 - \alpha)\, n_{\alpha}$ was determined from the slope of E_p versus $\ln\nu$ of equations (8) as 0.984. The electron transfer coefficient α for the oxidation of *Geshoidin* was determined (α = 0.49) from the Tafel slope of a linear scan voltammogram recorded at low scan rate (5 mV s^{-1}) [33]. Hence, the value of n_{α} was estimated to be 1.96 which is very close to 2. The $(1 - \alpha)n_{\alpha}$ value was then inserted into equation (9) and the

diffusion coefficient was determined for 5 x 10^{-5} mol dm^{-3} *Geshoidin* to be 5.80 x 10^{-6} cm^2 s^{-1}.

The variation of scan rate for the reduction peak (figure not shown) showed a shift in the peak potential towards negative values with increasing scan rate. The peak currents were also proportional to the square root of the scan rate. The peaks for the reduction, however, became ill-defined at high scan rates due to overlaps with the peak of the supporting electrolyte.

3.5.2. Influence of pH of the supporting electrolyte

The influence of pH on the oxidation peak current and oxidation peak potential of the cyclic voltammogram of *Geshoidin* was investigated over the range of pH 2 - 11. Figure 10 shows the dependence of the peak current on pH for three different concentrations of *Geshoidin*. The magnitude of the peak current for the respective concentration remained constant in the pH range 2 – 6 and then decreased until a minimum value of about pH 9 due to possibly the weak adsorption of the oxidized species. Beyond this pH, the current increased sharply. Since the magnitude of current is directly proportional to the rate of the electrochemical reaction [30, 32], it is apparent to conclude that rate of oxidation of *Geshoidin* is very high at higher pH. Figure 10 still shows that the peak current increases with the concentration of Geshoidin.

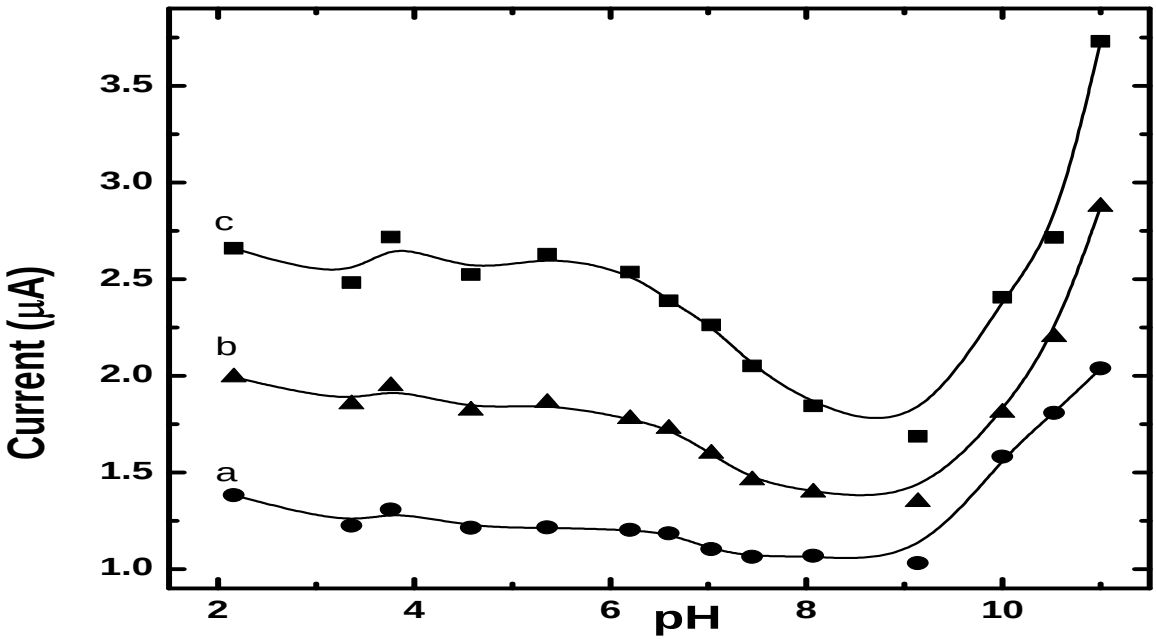


Figure 10: Plots of peak current as a function of pH for different concentrations of *Geshoidin*: (a) 5 x 10^{-5}; (b) 8 x 10^{-5}; (c) 1.0 x 10^{-4} mol dm^{-3} at a scan rate of 20

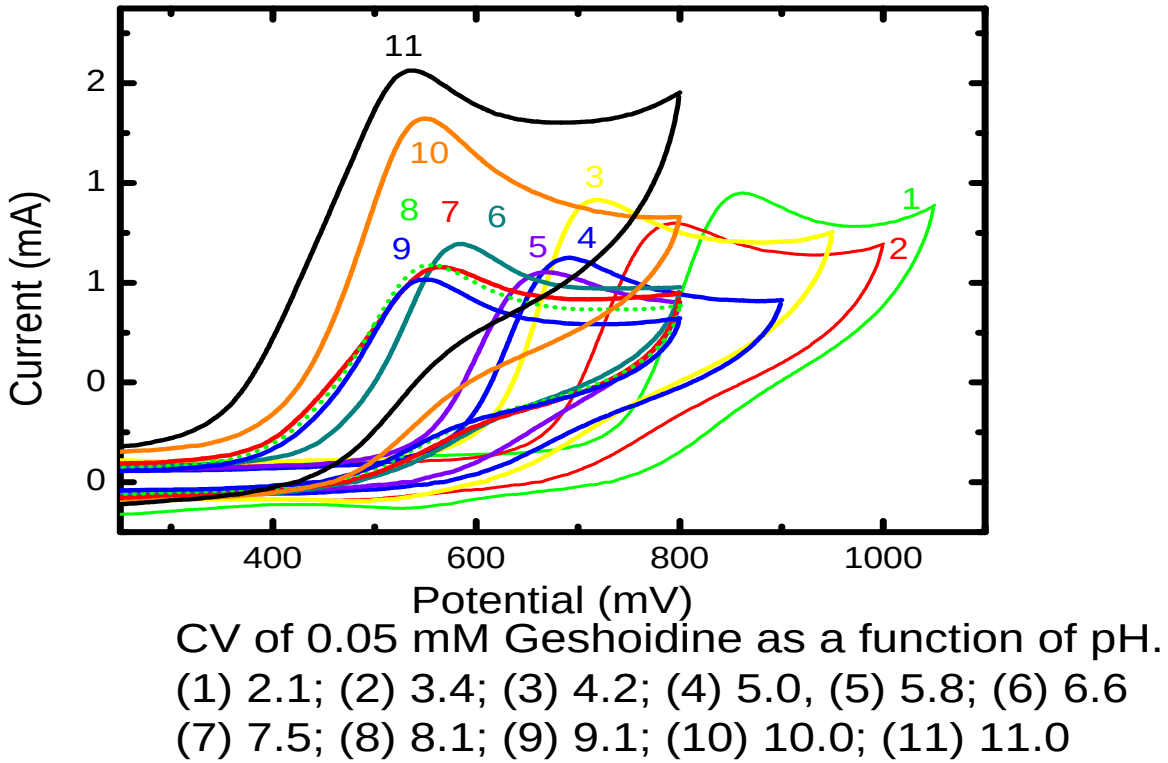


Figure 11: CV voltammogram of 0.05 mM Geshoidin as a function of the pH (2.1 to 11.0).

The shift in the oxidation peak potential as a function of pH was studied and linear dependence was observed. When the pH of the supporting electrolyte was increased the peak of the voltammograms was shifted to a more negative potential (figure 12).

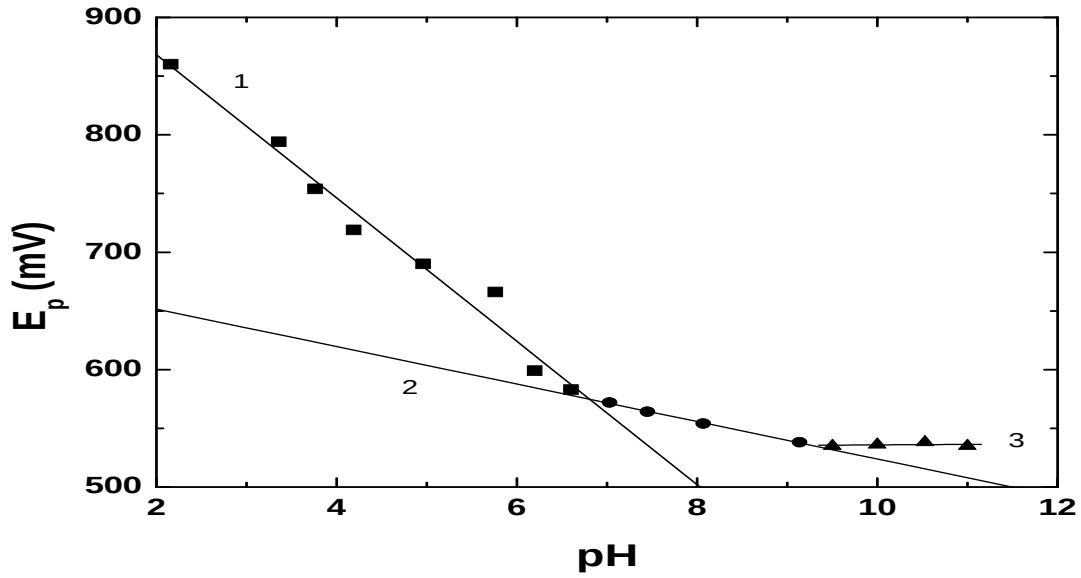


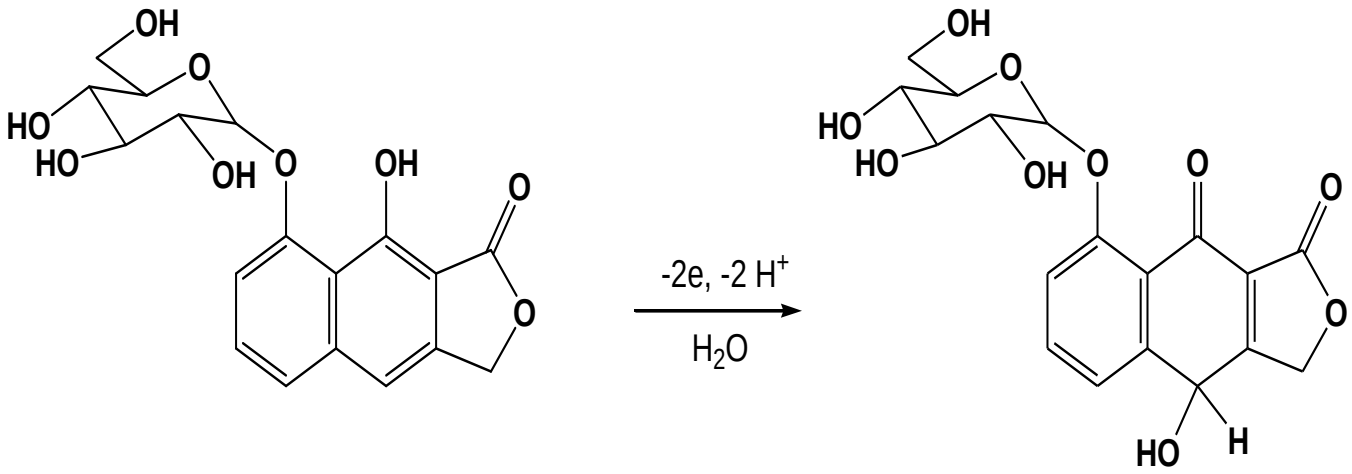
Figure 12: Plot of peak potential as a function of pH for the cyclic voltammetric oxidation peak of 5 x 10^{-5} mol dm^{-3} *Geshoidin* at a scan rate of 20 mV s^{-1}.

Two regions of linear dependences were observed. This indicates that the H^+ ion takes part in the electrode reaction. According to the literature [35], $E_p = K - (0.059y/n)pH$, where y is the number of H^+ ions that take part in the electrode reaction and n is the number of electrons. As can be seen from Fig. 12, there are two linear ranges, which are described by the following equations:

$E_P/V = 0.061pH + 0.990.$ $r^2 = 0.990$ (pH 2.0 – 7.0) (for n=7) ……. (5)
$E_P/V = 0.015pH + 0.683.$ $r^2 = 0.999$ (pH 7.2 - 9.2) (for n=4) …….. (6)

The peak potential is independent of pH and remains constant above pH 9.2. The dependence of the peak potential on pH has slopes of 61 and 15 mV per unit pH, respectively. This implies that the ratio of the number of protons involved changes from two (for n = 2) to zero as the pH becomes very high. Electrode processes involving a weak acid or weak base have a potential-pH variations which show a change in slope at pH = pK_a [33]. The oxidation behaviour of *Geshoidin* is strongly pH dependent and from the intersections of the linear parts of the plots of Fig. 12, the pK_a of *Geshoidin* was estimated as pK_{a1} = 6.81 and pK_{a2} = 9.27.

Organic compounds whose oxidation potentials are pH dependent undergo deprotonation reaction during oxidation [32]. The products obtained via phenol oxidation involve the formation of phenoxonium ions as intermediates. Phenoxonium ions are highly electrophilic species that react with a nucleophilic solvent such as water to give quinones and substituted quinones [36]. Below pH 7.2 it is apparent that two H^+ ions are removed from a molecule of *Geshoidin*. The fact that two electron oxidation wave is obtained and two hydrogen ions are involved in the electrode reaction in neutral and acidic solutions leads to the proposal of the following oxidation mechanism (Scheme 1) for *Geshoidin* at the electrode [17].



Scheme 1

3.5.3. Voltammetric analysis, linear range and detection limit of Geshoidin

Although the optimum oxidation current response of *Geshoidin* is achieved at very high pH (pH >10), performing electrochemical measurements at high pH is not desirable and hence a neutral pH was chosen for the analysis of Geshoidin in plant extracts. Using pH 7 and linear scan voltammetry the peak current was linearly dependent on *Geshoidin* concentration. Linear scan voltammograms at different concentrations of *Geshoidin* are shown in Figure 13.

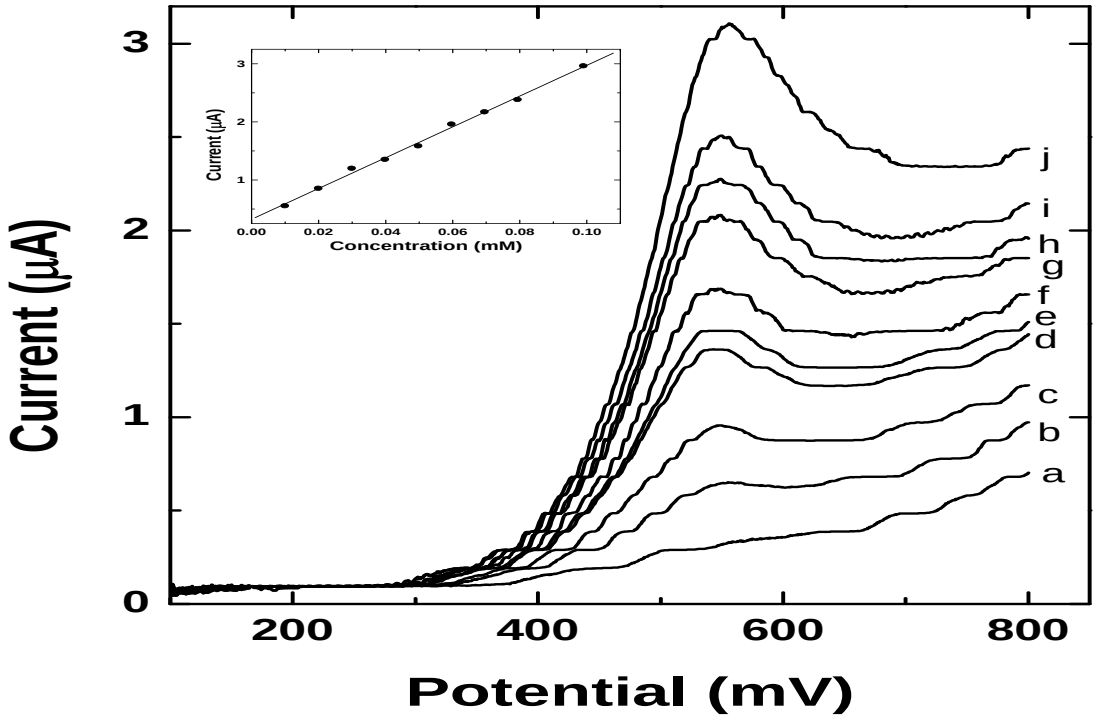


Figure 13: Selected linear scan voltammograms of *Geshoidin* for different concentrations of *Geshoidin*: (a) 0.1 mol dm^{-3} citric acid and 0.2 mol dm^{-3} di-sodium hydrogen phosphate buffer (pH 7); (b) 1 x 10^{-5}; (c) 2 x 10^{-5};(d) 3 x 10^{-5}; (e) 4 x 10^{-5}; (f) 5 x 10^{-5}; (g) 6 x 10^{-5}; (h) 7 x 10^{-5}; (i) 8 x 10^{-5}; (j) 1 x 10^{-4} mol dm^{-3}.

The dependence of the peak current as a function of concentration of *Geshoidin* is also shown in Figure 13 as an inset. Each data point in Figure 13 of the inset is the mean value of the peak currents obtained from three linear scan voltammetry runs. The peak current increased with increasing concentration of *Geshoidin*. The response was found to be linear in the concentration range 1.00 x 10^{-6} - 1.00 x 10^{-4} mol dm^{-3} *Geshoidin* and the correlation coefficient was r^2 = 0.999. At higher concentrations (≥ 3.00 x 10^{-4} M) deviation from linearity occurred due to saturation of the electrode surface. The detection limit (three times signal-to-noise ratio) was found to be 5.00 x 10^{-7} mol dm^{-3} *Geshoidin*. For eight successive

determinations of 1 x 10^{-5} mol dm^{-3} *Geshoidin*, a relative standard deviation (RSD) of 3.2% was obtained.

4. CONCLUSIONS.

The extraction of Geshoidin from the leaves of Rhamnus Prinoides L 'Herit was successfully performed over the period of the week and obtained 78.0g of the extract from 250g of the powdered leaves. 9.95g after subjecting into the preparative column chromatography resulted into a slightly impure brownish tinge precipitate of the isolated Geshoidin with the melting point range of 157 – 161 ^{0} C and the mass quantity of 36.8mg out of 9.95g of the extract.

The cyclic voltammetric responses obtained were chemically irreversible over the range of scan rates employed, and were consistent with electron transfer being followed by fast chemical process. The voltammetric method gave wide linear range for the determination of *Geshoidin*. Successful application of linear scan voltammetry for the determination *Geshoidin* in methanol extract from the leaf of the plant was demonstrated.

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