Effect of *Ocimum sanctum* (Tulsi) leaf extract on prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) in human plasma

By

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Declaration

I hereby declare that I am the sole author of this thesis titled as 'Effect of *Ocimum sanctum* (Tulsi) leaf extract on prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) in human plasma'. I declare that this thesis is being submitted to Universiti Sains Malaysia (USM) for the purpose of the award of Master of Science in Transfusion Science. This dissertation is the result of my own research under the supervision of Dr.Siti Salmah and Dr.Muggundha Raoov except as cited in the references. The dissertation has been accepted for the study performed and is not concurrently submitted in candidature of any other degree.

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List of Abbreviations

i. AMDI : Advanced Medical & Dental Institute
ii. APTT : Activated Partial Thromboplastin Time
iii. CVA : Cerebral Vascular Accident
iv. GCMS : Gas Chromatography Mass Spectrometry
v. HMWK : High-molecular- weight-kininogen
vi. NIST : National Institute Of Standard and Technology
vii. NRCS : National Resources Conversation Service
viii. PPP : Platelet Poor Plasma
ix. PT : Prothrombin Time
x. TFPI : Tissue Factor Inhibitor
xi. TT : Thrombin Time

List of Symbols

i. % : Percentage
ii. µm : micrometer
iii. dH_2O : Distilled water
iv. g : gram
v. m : meter
vi. ml : millimeter
vii. NaCl: Sodium Chloride
viii. ℃ : Degree Celcius
ix. Rpm : rotation per minutes
x. w/v : weight / volume
xi. µg : microgram

Abstrak

Walaupun, terapi antikoagulan konvensional terbukti berkesan dalam trombosis vena dalam dan embolisme pulmonari namun terdapat beberapa kelemahan dalam terapi ini seperti lebam, sakit, bengkak atau batuk berdarah. Oleh itu, usaha untuk menghasilkan antikoagulan yang novel yang berasal daripada bahan-bahan semula jadi seperti tumbuh-tumbuhan dituntut pada masa kini. *Ocimum sanctum* juga dikenali sebagai *Ocimum tenuiform* (OT), Tulsi atau selasih suci yang berasal daripada keluarga Lamiaceae telah digunakan secara meluas untuk beribu-ribu tahun dalam perubatan Ayurveda dan sistem Unani untuk menyembuhkan atau mencegah beberapa penyakit seperti sakit kepala, demam malaria, ulser, bronkitis, batuk, selesema, sakit tekak dan asma. Tujuan kajian ini adalah untuk menyiasat kesan ekstrak daun *Ocimum sanctum* (Tulsi) terhadap prothrombin time (PT), activated partial tromboplastin time (APTT) dan thrombin time (TT) dalam plasma manusia. Aktiviti pembekuan *O.sanctum* disiasat melalui ujian PT, APTT dan TT dalam plasma citrated yang diperolehi daripada tiga puluh enam penderma darah yang sihat. Plasma ini diuji dalam beberapa kepekatan *O.sanctum* iaitu 0.1mg/ml, 0.5mg/ml dan 1.0mg/ml. Hasil kajian mendapati ekstrak akueus *Ocimum sanctum* menunjukkan masa yang lebih panjang bagi ujian PT dan APTT (p <0.05) tetapi tidak memberi kesan kepada ujian TT (p> 0.05). Analisis GCMS telah mendapati asid linolenik pada 1-10% etanol dan akueus di masa tahanan yang berbeza yang bertanggungjawab bagi aktiviti-aktiviti pembekuan *Ocimum sanctum* dalam plasma manusia. Kesimpulannya, kajian ini menunjukkan bahawa *Ocimum sanctum* menjejaskan aktiviti pembekuan dalam plasma manusia dan boleh berpotensi digunakan sebagai produk antikoagulan semulajadi yang boleh diperolehi pada masa depan.

Abstract

Even tough, the conventional anticoagulant therapy proven to be effective in deep vein thrombosis and pulmonary embolism but there are several drawbacks such as bruises, pain, swelling or coughing blood. Therefore, the search for novel anticoagulant derived from natural substances like plants are demanded nowadays. *Ocimum sanctum* also known as *Ocimum tenuiform* (OT), tulsi or holy basil from the family of Lamiaceae has been widely used for thousands of years in Ayurveda and Unani system to cure or prevent a number of illness such as headache, malaria fever, ulcers, bronchitis, cough, flu, sore throat and asthma. The aim of this study is to investigate the effect of *Ocimum sanctum* (Tulsi) leaf extract on prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) in human plasma. The coagulation activity of *O.sanctum* was measure via PT, APTT and TT assay in citrated plasma collected from thirty-six healthy regular blood donors. The plasma was tested against different concentration of *O.sanctum* aqueous extract as follows 0.1mg/ml, 0.5mg/ml and 1.0mg/ml. The result showed, the aqueous extract of *O.sanctum* prolonged the PT and APTT assays ($p<0.05$) but no affect on TT assay ($p>0.05$). The GCMS analysis had identified the linolenic acid at 1-10% of ethanol and aqueous concentration at different retention time which was responsible for the coagulation activities of *O.sanctum* in human plasma. In conclusion, this study suggests that *O.sanctum* does affect coagulation activity in human plasma and can be potentially used as naturally derived anticoagulant products in future.

CHAPTER 1

Introduction

1.1 Blood Coagulation

Haemostasis is the normal physiological process of blood clotting which involves dissolving and lysing of clotted blood. Haemostasis can be divided into two major systems as follows; primary and secondary haemostasis (Abdel-Wahab *et al.,* 2006). These two processes happen concurrently and interrelated. The primary haemostasis is a short lived which involves platelets and vascular response to vessel injury which lead to the formation of initial 'platelet plug'. The vascular wall is lined with endothelial cells which show antithrombotic properties due to coagulation inhibitors, neutral phospholipids and fibrinolysis activators (Anshu *et al.,* 2014).

On the other hand, the sub endothelial layer contains collagen and laminin which is highly thrombogenic. As a result, vasoconstriction slows the blood flow at the damaged blood vessel to limit the blood loss which usually last up to 30 minutes. These process is mediated by two controls; local and systemic controls. The local controls are the vasoconstrictors such as thromboxane (produced at injured site) whereas systemic control is the epinephrine produced by adrenal glands to stimulate vasoconstriction (Cohen, 2014). In addition, vasoconstriction enhances the platelet adhesion and activation which in turn lead to platelet accumulation and prevent blood loss.

Meanwhile, the secondary haemostasis is the formation of insoluble fibrin which is produced via proteolytic coagulation cascade. These insoluble fibrin meshes were then

incorporated into the platelet plug formed during primary haemostasis which will enhance and stabilize the blood clot (Gale, 2011). Secondary haemostasis involves coagulation factors and fibrinolytic system in respond to injury.

Blood coagulation is a complex process that forms blood clots to block/heal a lesion which involves cellular (platelet) and protein (coagulation factors). Mostly the coagulation factors are precursors of proteolytic enzymes, zymogens which circulate in an inactive form and a suffixing letter 'a' will be given to the activated zymogen (Anshu *et al.*, 2014). Clotting factors are classified into three groups as follows; fibrinogen family, Vitamin K dependent proteins and contact family (Table 1.1). In addition, coagulation process is control by inhibitor (plasminogen & protein C) which disrupt the clot formation as well as development of thrombus.

Fibrinogen family	Vitamin K dependent	Contact family
Fibrinogen	Factor II	Factor XI
Factor V	Factor VII	Factor XII
Factor VIII	Factor IX	HMWK
Factor XIIII	Factor X	Prekallikerin

HMWK – High molecular weight kininogen

Table 1.1: Classification of coagulation factors. (Anshu *et al.*, 2014)

Blood coagulation system can be divided into three pathways; extrinsic, intrinsic and common pathways which are enzymatic cascade that is activated upon tissue damage (Rehan *et al.*, 2001).

1.1.1 Extrinsic Pathway

Based on figure 1.1, extrinsic pathway involves vascular and blood components which are activated upon the release of tissue factor/tissue factor pathway (Factor III) at the site of injury and takes up to 10-15seconds to complete the entire process (Dorothy *et al.,* 2012). Tissue factor is a glycoprotein which is found on surface of subendothelial tissue and exposed during vascular injury. Similar to activation of factor IXa in intrinsic pathway, tissue factor act as a cofactor for Factor VIIa which contain serine protease that cleaves factor X to Factor Xa in the presence of calcium ions. The association between factor VIIa and tissue factor is the principal step in clotting cascade. Next, factor Xa and factor Va convert prothrombin/factor II to thrombin which then cleaves fibrinogen to fibrin. Furthermore, inhibition of extrinsic pathway by Tissue Factor Pathway Inhibitor (TFPI) occurs at factor VIIa-Xa complex (Gale 2011). TFPI also known as lipoprotein-associated coagulation inhibitor (LACI) consist of 3 protease inhibitor domains as follows, domain 1 will bind to factor Xa whereas domain 2 binds will to factor VIIa with the presence of factor Xa (Anshu *et al.,* 2014).

1.1.2 Intrinsic Pathway

Meanwhile, the intrinsic/contact pathway is activated when the blood contact with the negatively charged substances such as endothelial surface,prekallikrein, collagen, phospholipids or high-molecular-weight kninogen (HMWK)(Figure 1.1). Factor I, II, V, VIII, IX and XII plays an important role in these pathway (Anshu *et al* 2014). Upon conversion of prekallikrein to kallikrein, it activates factor XII to XIIa which then activates factor XI to XIa. Factor XIa then activates factor IX (vitamin K dependent) to IXa in the presence of calcium ions. Factor VIIa and Factor IXa forms complexes with phospholipid which then activate factor Xa. Similar to extrinsic pathway, activation of Factor Xa convert prothrombin to thrombin which then cleaves fibrinogen to fibrin. Furthermore, the production of thrombin can be controlled via negative feedback with the activation of Protein C (Gale, 2011). Protein C binds to thrombomodulin and promotes the degradation of factor Va and VIIIa which then inhibits the activation of prothrombin.

1.1.3 Common Pathway

The common pathway is the meeting point of extrinsic and intrinsic pathway in which factors I, II, V and X takes part. Factor Xa convert prothrombin to thrombin which then cleaves fibrinogen to fibrin. The generation of Factor V, VIII, Thrombin, antithrombin and Protein C will either enhance or inhibit thrombin production via positive or negative feedback. In positive feedback regulation, factor Va and VIIIa will enhance thrombin production by activating prothrombin or factor Xa (Dorothy *et al.*, 2012). Meanwhile, in negative feedback, Protein C will bind to thrombomodulin and promotes the degradation of factor Va and VIIIa which then inhibits the activation of prothrombin (Gale, 2011). Thus, these regulations prevent excessive blood loss which could lead to stroke or heart attack.

1.1.4 Fibrinolytic system

The final step in haemostasis involves the fibrinolytic system which is parallel to the activation of coagulation cascade. Fibrinolytic system involves primarily three serine proteases enzymes which dissolves fibrin clot into fibrin degradation products (FDPs) (Raber,1990). Plasmin is produced from inactive plasminogen in liver by proteases tissue-type plasminogen activator (TPA) as well as urokinase-type plasminogen activator (uPA). Plasmin activity is inhibited by α-2 antiplasmin and α-2Macroglobulin which prevent fibrinolysis (Dorothy *et al.*, 2012). Meanwhile, plasminogen activator inhibitor main inhibitor of fibrinolysis which act by inhibiting t-PA and u-PA (Anshu *et al.*,2014) In addition, thrombin activatable fibrinolysis inhibitor (TAFI) which is a proenzyme produced by liver and is activated by thrombin act by reducing the affinity of plasminogen to fibrin (Bouma & Mosnier, 2006).

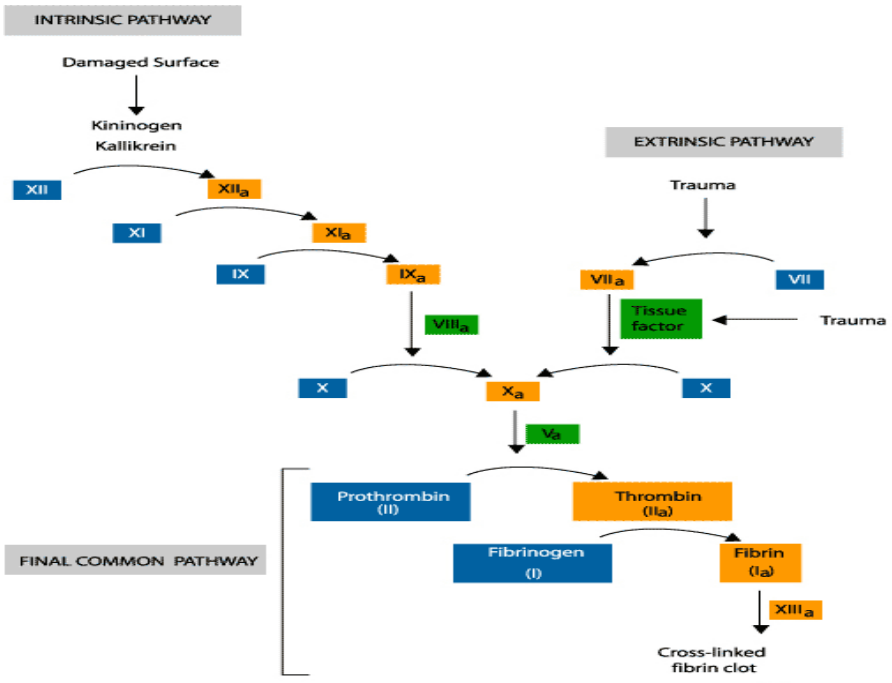

Figure 1.1: The overall blood clotting cascade (Crooks and Hart 2015)

1.2 Haemostasis Screening Assay

Prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) are used to detect the blood coagulation disorder. PT assay measure the deficiencies in the extrinsic and common pathway but does not measure Factor XIII activity or intrinsic pathway (Behera, *et al.*, 2010). Meanwhile, the APTT measures the intrinsic and common pathways but does not measure factors VII and XIII. The TT assay measures the rate of conversion of fibrinogen to fibrin.

1.2.1 PT Assay

PT measure the time taken to produce fibrin upon the activation of factor VII. In PT assay, citrated plasma is used together with an activating agent; thromboplastin prepared from rabbit cerebral tissue (Raber,1990). The plasma is incubated at 37 °C and the times taken for the fibrin filaments to be formed are measured. The normal reference range for PT assay is between 12.2 -14.2 sec. If there is deficiency in coagulation factor or presence of coagulation inhibitor, PT will be prolonged. Thus, the test is repeated with 1:1 mix with normal plasma. PT assay is more sensitive in detecting in factor deficiencies than APTT (Dorothy *et al* 2012). PT is important in detecting inherited or acquired deficiencies. Inherited deficiency of factor VII will have prolonged PT which is a rare bleeding disorder, but PT become normal when mixed with normal plasma (1:1). Meanwhile, acquired deficiencies are due to warfarin therapy or liver disease.

1.2.2 APTT Assay

APTT is also known as Kaolin Cephalin Clotting time (KCCT) or Partial Thromboplastin Time with kaolin (PTK). APTT is used to test for inherited or acquired factor deficiencies. In inherited disorder such Hemophilia A (FVIII deficiency) and Hemophilia B (FIX deficiency), APTT will be prolonged (Gale, 2011). Meanwhile, acquired factor deficiency can be due to liver dysfunction and vitamin K deficiency which are more common. APTT measure the time taken to produce fibrin upon the activation of intrinsic pathway. A prolonged APTT will be seen if any of these factors, prothrombin, fibrinogen, factors V, VIII, IX, X, XI and XII are abnormal (Cohen, 2014). In laboratory, citrated plasma, cephalin, an activating agent (kaolin) and calcium ions are added together and followed by incubation at 37 °C (Anshu *et al.*,2014). The time taken for the clumping of kaolin will be measured. Kaolin is surface activator which binds to FXII and activates to FXIIa. Meanwhile, cephalin is a phospholipid derived from rabbit cerebral tissue, used to replace platelet phospholipids which are necessary for the normal function of coagulation cascade.

The normal range for APTT is 31.7-44 sec. A prolonged APTT can be seen either in the presence of inhibitor for intrinsic pathway or reduced activity of the factors (<30%). This can be further differentiated via *mixing study* which uses normal and patient plasma in a ratio of 1:1. If the result is completely corrected, then the prolonged APTT is due to factor deficiencies. Meanwhile, if the result is still abnormal, then the prolonged APTT is due to presence of inhibitor such as antithrombins which disrupt the activity of thrombin in conversion of fibrinogen to fibrin (Raber, 1990). However, APTT will shows normal result if the factor is approximately 50%.

1.2.3 TT Assay

TT assay measures the time taken for the conversion of fibrinogen to fibrin in the presence of thrombin. Usually TT assay tests for the acquired deficiency of fibrinogen which is due to coagulopathy or severe liver disease. In this test, citrated plasma and thrombin are added together then incubated at 37 °C (Raber, 1990). The time taken for the formation of fibrin filaments is measured. The normal range for TT assay is 15-20sec. Abnormalities of TT assay can be due to three reasons. First, it can be due to deficiency in volume of fibrinogen (<100 mg/dl) (Choi *et al.,* 2003). In addition, it also can be due to the presence of inhibitor (heparin or fibrin degradation products, FDP) or abnormal fibrinogen. Similar to APTT and PT, *mixing study* can be performed in 1:1 ratio to distinguish between deficiency of fibrinogen or decrease in fibrinogen.

1.3 Blood Coagulation Disorders

Blood coagulation factors and platelet are the two crucial components to maintain haemostatis. Bleeding disorder can be inherited or acquired and occur due to deficiency to any of the components. Examples of inherited bleeding disorder are von Willebrand's disease and haemophilia A whereas example of acquired bleeding disorder is liver disease. These disease can be treated with antifibrinolytic or procoagulant to enhance coagulation cascade. In addition, the formation of blots/thrombus (thrombosis) can also lead to various diseases such as pulmonary embolism and cerebral vascular accident (CVA) (Pandey and Madhuri, 2010). However, anticoagulant therapy can be given to prevent thrombus formation. Anticoagulant can be obtained by two ways; conventional therapy (drug) or natural/herbal medicine.

There are number of drugs used as anticoagulant; vitamin K antagonist (VKA), non-VKA (Apixaban), (Caumadin) and heparin (Enoxaparin and Porcine) .Vitamin K antagonist are drugs that reduce fibrin production by manipulating vitamin K activity. Warfarin is an example of oral VKA that inhibits the enzyme, Vitamin K epoxide reductase which involves in oxidation of vitamin K (Mphil *et al.,* 2014). Since factor II, VII, IX and X requires vitamin K to be activated, thus by reducing the production of vitamin K will prevent the formation for thrombin (Grover *et al.,* 2005).Non -VKA also known as novel oral anticoagulant (NOAC) such as apixaban and edoxaban are new alternative drugs which directly binds to thrombin. These drugs have wide therapeutic window (Bauer, 2013).

On the other hand, Heparin can be divided into two types as follows; unfractionated heparin (UFH) and low molecular weight heparin (LMWH) (Hirsh & Rashke, 2004). UFH

is a mixture of sulphated glycosaminoglycans with a molecular weight of 3,000-30,000 daltons. UFH (Porcine) is an intravenous anticoagulant that activates antithrombin III followed by the inactivation of thrombin and factor Xa involves in blood clotting cascade (Anand *et al.,* 2001). Meanwhile, LMWH is obtained from unfractionated heparin via enzymatic and chemical depolymerization with a molecular weight 4,500-5,000 dalton. Similar to UFH, LMWH also activates antithrombin III which is mediated by pentasaccharide sequence but with a lower affinity.

Even tough, the conventional therapy proven to be effective in deep vein thrombosis and pulmonary embolism but there are several drawbacks such as bruises, pain, swelling or coughing blood. The main adverse effect associated with warfarin is bleeding which is about 7.2 and 1.3 per 100 patient-years according to meta- analysis by Choi *et al.,* (2003). However, for a newer anticoagulant such as apixaban and edoxaban , these drugs have a wider therapeutic window which make the laboratory monitoring difficult. In addition, these drugs have uncertainty of drugs dosing in some patient populations such as patient with underlying chronic disease (Bauer, 2013).

Therefore, the study of natural substances with anticoagulation properties that present in vegetables and plants has been widely explored to replace the currently available anticoagulant drugs. One of the natural substances that have anticoagulant properties is linolenic acid which is present in *Ocimum sanctum*. Hence this study is performed to look at the basic coagulation screening which is PT, APTT and TT at various aqueous concentration of *Ocimum sanctum in vitro*.

1.4 Objectives

1.4.1 General Objectives

- To study the effects of *Ocimum sanctum* leaves extracts on human blood coagulation activities *in vitro.*

1.4.2 Specific Objectives

- To determine the effects of different aqueous extract concentration of *Ocimum sanctum* leaves on prothrombhin time (PT) activated partial thromboplastin time (APTT) and thrombin time (TT) assays.
- To identify the linolenic acid present in *Ocimum sanctum* leaves in aqueous and ethanol extract that involves in blood coagulation activities via Gas Chromatographic Mass Spectrometry (GCMS).

1.5 Hypothesis

H_A: *Ocimum sanctum* leaves enhance the blood coagulation activities of human in *in vitro*.

H_0: *Ocimum sanctum* leaves have no affects on blood coagulation activities.

1.6 Expected outcome

There are blood coagulation activities in aqueous extract of *Ocimum sanctum* which can be determined using PT, APTT, TT assays *in vitro.* This outcome benefits in clinical treatment as it possess procoagulant or anticoagulant. If the *Ocimum sanctum* acts as anticoagulant (prevent blood clotting) then, it can be used for treatment of thrombosis such as pulmonary embolism and deep vein thrombosis. Meanwhile, if *Ocimum sanctum* acts as procoagulant (promotes blood coagulation) then it can be used to treat acute bleeding and hemorrhage The other expected of this study is to look at compositions of *Ocimum sanctum* that enhance blood coagulation activities which can be identified in ethanol and aqueous extract using GCMS. If this is presence, this OS can be potentially used as alternative anticoagulant/procoagulant agents.

CHAPTER 2

LITERATURE REVIEW

2.1 *Ocimum sanctum*

Ocimum sanctum (OS) (figure 2.1) also known as *Ocimum tenuiform* (OT), tulsi or holy basil from the family of Lamiaceae has been widely used for thousands of years in Ayurveda and Unani system to cure or prevent a number of illness such as headache, malaria fever, ulcers, bronchitis, cough, flu, sore throat and asthma (Jung *et al.*, 2002). Tulsi, the 'Queen of herbs' can be divided into three types; Rama/light tulsi (OS), Shyama/Dark tulsi (OS) and Vana tulsi (OT) which have similar chemical properties and medicinal values.

Figure 2.1: *Ocimum sanctum*

2.1.1 Structure

OS is an erect plant, 30-60cm tall with green/purple leaves that are fragrant and hairy stems. The aromatic leaves are oblong, ovate, petiole, sharp point or blunt and toothed or non toothed which grow up to 5cm long (Kadian and Parle, 2012). Stems and branches are purplish in color, sometimes woody below. Meanwhile, the flowers are elongate racemes and small in size. The seeds are flat and reddish-yellow in color. The fruits are small in size.

2.1.2 Classification

Scientific classification:

Rank	Scientific Name and Common name
Kingdom	Plantae-Plants
Subkingdom	Tracheobionta-Vascular plants
Superdivision	Spermatophyta-seed plants
Division	Magnoliophyta-Flowering plants
Class	Magnoliopsida-Dicotyledons
Subclass	Asteridae
Order	Lamiales
Family	Lamiaceae –Mint family
Genus	*Ocimum* L.-basil
Species	*Ocimum sanctum* L- holy basil

Adapted from Natural Resources Conservation Service, United States Department of Agriculture

2.1.3 Geographical Distribution

OS is widespread as cultivated plant for medicinal/ religious purposes and distributed all over the world tropics (Madhuri and Pandey , 2010). OS is grown in India from Andaman and Nicobar islands up to 1800m above the sea level of Himalayas (Ali *et al.*,2006). In addition, it also found in Australia, West Africa, Malaysia and some part of Arab countries.

2.1.4 Active Compounds

2.1.4.1 Phytoconstituents

Based on table 2.1, the phytochemicals presents in OS can be obtained from various part of the plant via fixed oil, essential oil, mineral contents and alcoholic extract. The leaves extract of OS consist of 0.7% of volatile oil which includes 71% of eugenol (major component) and 20% methyl eugenol (Kadian and Parle, 2012). In addition, the oil also contains sesquiterpine hydrocarbon caryophyllene and carvacrol. Meanwhile, stem and leaves extract of OS will yield phenolic compounds such as apigenin, circimaritin and rosameric acid (Gautam and Goel, 2014). On the other hand, linoleic acid, linolenic acid and stearic acid can be obtained from seeds via fixed oil extracts. In addition, vitamin A, calcium, copper and iron are derived from the entire plant via extract of mineral contents.

Table 2.1: Phytochemical constituents of *Ocimum sanctum* (Kadian and Parle, 2012)

No/s	Extract	Phytochemicals	Plants Parts
1	Fixed oil	Linoleic acid, Linolenic acid, Oleic acid, Palmitric acid, Stearic acid.	Seeds
2	Essential oil	Aromadendrene oxide, Benzaldehyde, Borneol, Bornyl acetate,Camphor, Caryophyllene oxide, cis-Terpineol, Cubenol,Cardinene, D-Limonene, Eicosane Eucalyptol, Eugenol,Farnesene, Farnesol, Furaldehyde, Germacrene, Heptanol,Humulene, Limonene, n-butylbenzoate, Ocimene, Oleic acid, Sabinene, Selinene, Phytol, Veridifloro, _-Camphene, _-Myrcene, _Pinene, _-Pinene, _-Thujene, _-Guaiene, _-Gurjunene, methyl chavicol and linalool.	Leaves
3	Mineral contents	Vitamin C, Vitamin A, Calcium, Phosphours, Chromium, Copper,Zink, Iron.	Whole Plant
4	Alcoholic extract	Aesculectin, Aesculin, Apgenin, Caffiec acid, Chlorgenic Acid,Circineol, Gallic Acid, Galuteolin, Isorientin, Isovitexin, Luteolin, Molludistin, Orientin, Procatechuic acid, Stigmsterol,Urosolic acid, Vallinin, Viceni, Vitexin, Vllinin acid.	Leaves/areal parts

2.1.5 Medicinal Values

2.1.5.1 Pharmacological activities

There are number of pre clinical studies in animals' models and *in vitro* testing that have been carried out to report the pharmacological activities of OS. It has been reported that OS leaves extract in petroleum, ethanol and aqueous have wound healing activity, anticancer, antidiabetic, antioxidant, anti-inflammatory and anticoagulation properties.

2.1.5.2 Wound Healing Activity

In a study done by Shetty *et al*., (2006), the effect of aqueous extract of OS in wound healing on rats has been investigated. In this study, the albino rats were divided into two groups irrespective to sex, group 1: wounded control rats whereas group 2: wounded rats given OS aquoeus extract. The wound healing had been monitored via wound breaking/contraction strength in incision/excision wound model and epithelization period. The result showed an increase in percent of wound contraction in rats given with OS extract. Thus, OS can be used to manage any abnormal healing such hypertropic scars.

Similarly, another study done by Shetty *et al* in 2006, the ethanolic extract of OS leaves were used to investigate normal wound healing versus dexamethasone depressed wound model. The result showed that, the extract increases the wound breaking/contraction strength and wound epithelializes rapidly as compared o dexamethasone. Thus, it can be said that OS ethanolic extract promotes wound healing as well as overcome the suppression action of dexamethasone (Shetty *et al*., 2006).

2.1.5.3 Anticancer

In 1999, Govindasamy and his colleagues have done an investigation regarding chemopreventive effect of OS versus 7,12-dimethylbenz (a) anthracene (DMBA) on hamster buccal pouch carcinogenesis. In this study, OS was given in three forms; aqueous extract, fresh leaf paste and ethanolic extract. OS was given orally and topically applied to the buccal pouch of hamster which was exposed to 0.5% DMBA. As a result, the production of squamous cell carcinoma and papillomas were reduced and thus increase the survival rate. In addition, the aqueous extract showed higher chemopreventive effect than the other two forms which was proven via histopathological observations.

Similarly, Gupta and Prakash (2000) investigated the chemopreventive activity of OS seed oil versus 20-methylcholanthrene induced tumors in the thigh of albino mice. As a result, the chemopreventive effect and antioxidant properties of OS seed oil disrupt tumor production and thus enhance survival rate of albino mice.

2.1.5.4 Antidiabetic

The oral administration of OS ethanolic extract has been reported to reduce blood sugar level in normal, urea and glucose fed hyperglycemic with marked increases in insulin and glycogen tolerance in streptozotocin-induced diabetic rats (Kandasamy *et al.,* 2006). In addition, the use OS ethanolic extract has also increase the production of C-peptide. In another study done by Abdel-Wahab *et al.,* (2006) which used four types of OS extract butanol, aqueous, ethylacetate and ethanol which were studied on the insulin production as well as mechanism of action of every extract. As a result, it shows that ethanol extract stimulate the production of insulin from isolated islets, perfused pancreas and clonal pancreatic beta cells but the other extraction decrease the production. In 2006, Ali *et al.,* reported that OS induce the production of insulin which reflects its antidiabetic property. However, the oral administration of OS does not have any effect on hyperinsulinemia (Grover *et al.,*2005).

2.1.5.5 Antioxidant:

Another property of OS is antioxidant which is due to the presence of flavonoids. In 2000, a study conducted by DeWitt *et al.,* stated that orientin and vicenin (flavonoids) *in vitro* were expressed in radiation induced lipid peroxidation in mouse liver. Therefore, the OS extract able to react with highly reactive free radicals. Meanwhile, the aqueous extract of OS has the ability to increase the antioxidant activity of superoxide dismutase which is an enzyme catalyze the reaction of superoxide to oxygen or hydrogen peroxide (Gupta *et al.,* 2006). On the other hand, Acharya *et al.,* (2004), has investigated the effect of methanolic extract of OS on hypoperfusion and cerebral reperfusion on rats. Thus, OS extract decreased the superoxide dismutase (SOD) activity as well as lipid peroxidation. In conclusion, OS can be used to treat cerebrovascular insufficiency as well as reperfusion injury.