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Ph.D. Dissertation

Chemical and Biochemical Evaluation in Five Varieties of *Piper betle* L. Leaves from Bangladesh

Graduate School of Chosun University

Department of Food and Nutrition

Md. Atikul Islam

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방글라데시산 *Piper betle* L. 잎의 품종별 화학적 평가

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Dedicated to

My parents

Who planted the seed of this Ph.D. dream in my mind

My wife and sisters

Who have supported me with their valuable opinions in my every decisions

My daughter and son

Who have sacrificed fatherly touch in their childhood to make a father's dream come true

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Symbol	Description
µ	Micro
AD	Anno Domini
AOAC	Association of Official Analytical Chemists
ABTS	2,2'-azino-bis-3-et hylbenzthiazoline-6-sulphonic acid
CCC	Center Circumstance Composite
DMSO	Dimethyl Sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
E	East
FBL	Five varieties of *Piper betle* L. leaves
g	Gram
GC-MS	Gas Chromatography-Mass Spectrometry
HCA	Hierarchical Cluster Analysis
HeLa	Human Epithelial Cell
ICP-MS	Inductively Coupled Plasma- Mass Spectrometry
ICP-OES	Inductively Coupled Plasma- Optical Emission Spectrometry
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kg	Kilogram
L	Liter
LDA	Linear Discriminant Analysis
LOD	Limit of Detection
LOQ	Limit of Quantification

Symbol	Description
mg	Milligram
MSCs	Mesenchymal Stem Cells
N	North
ºC	Degree Centigrade
PCA	Principal Component Analysis
RDI	Recommendation Daily Intake
RSM	Response Surface Methodology
SDE	Simultaneous Distillation Extraction
SH-SY5Y	Human Neuroblastoma Clonal Cell
SPSS	Statistical Package for Social Sciences
Var.	Varieties
WHO	World Health Organization

ABSTRACT

Chemical and Biochemical Evaluation in Five Varieties of *Piper betle* L. Leaves from Bangladesh

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Piper betle L. leaves have been extensively consumed for mastication, mouth freshening, and medicinal purposes throughout the world. These leaves still lacked data, especially on their quality, safety, and efficacy in Bangladesh. This study aimed at evaluation of chemical (elemental: major, minor, trace, and toxic element; volatile and non-volatile organic compounds) and biochemical (antioxidant, antimicrobial, and cytotoxicity) properties in five varieties of *Piper betle* L. leaves (FBL) var. Bangla, Sanchi, Misti, Khasia, and BARI Paan 3 from Bangladesh.

Inductively coupled plasma-optical emission spectrometry (ICP-OES) and inductively coupled plasma-mass spectrometry (ICP-MS) analysis of elemental concentrations in FBL were found to be decreasing order of elements (major: K > Ca > Mg > P > Na; minor: Mn > Fe > Sr > Ba > Zn > Cu > Cr > Ni; trace: Ga > Li > V > Cs > Se > U > Be; toxic: Pb > As > Cd > TI). Among the nutritionally important elements, all examined betel leaves were good sources of K (3.99 – 5.86 g/kg), Ca (2.64 – 2.80 g/kg), and Mg (1.26 – 1.33 g/kg). Whereas, potentially all minor and toxic elements were present in safe limit except Mn (21.94 – 25.09 mg/kg) and Pb (129.36 – 965.91 µg/kg) specified by World Health Organization (WHO) / Food and Agriculture Organization (FAO).

Simultaneous distillation extraction and gas chromatography-mass spectrometry (SDE/GC-MS) were applied to analyze the volatile organic compound in FBL. The betel leaf

variety Misti showed the highest amount of volatile organic compounds (13958.90 mg/kg) followed by BARI Paan 3 (11684.10 mg/kg), Khasia (11109.70 mg/kg), Sanchi (6958.51 mg/kg), and Bangla (4346.91 mg/kg). A total of 101 compounds were identified in this analysis. Out of those, 42 were common in all varieties with different quantities, and the other 59 compounds were not available in each variety. The present research reported 50 new volatile organic compounds in betel leaves for the first time compared to published literature. Eugenol was found in all varieties as a major compound and other main compounds were β-caryophyllene, γ-muurolene, valencene, eucalyptol, chavicol, and caryophyllene oxide.

The non-volatile organic compound screening tests confirmed the presence of different non-volatile organic compound classes like phenol, flavonoids, terpene, steroids, phytosterols, and saponin in FBL. Finding out the optimum parameters for the ultrasonic extraction from betel leaves using response surface methodology (RSM) with central composite circumscribed (CCC) design to maximize the total phenolic content. The solvent was selected as ethanol: acetic acid: water (70%: 5%: 25%, v/v) and the optimal extraction condition was: time 90 min; extraction temperature, 75 °C; solid/liquid ratio, 1:15.41. Followed by, FBL were extracted by the above optimized ultrasonic extraction conditions and their total phenolic and flavonoid contents showed high variation ranging from 110.51 to 322.8 mg GAE/g DW and 46.79 to 57.09 mg QE/g DW, respectively. The major phenolic compound hydroxychavicol was quantified 14.19 mg/g to 38.19 mg/g in FBL by high-performance liquid chromatography–diode array detector (HPLC-DAD) analysis.

The antioxidant activity of IC_{50} value of 2,2-Diphenyl-1-pycrilhydrazil (DPPH) and 2,2'-Azino-(bis 3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) assay was ranged from 0.17 to 0.43 mg/mL and 0.04 to 0.11 mg/mL, respectively, in FBL extract. The IC_{50} values of DPPH and ABTS assay were 10.88 to 14.47 µg/mL and 7.66 to 18.00 µg/mL for hydroxychavicol and eugenol, individually, which are the major phenolic compounds. The same extracts were displayed good inhibitory properties against *S. typhimurium*, *P. aeruginosa*, *E. coli*, *A. faecalis*,

and MRSA bacteria compared to other tested bacteria. Khasia and BARI Paan 3 were showed the highest antioxidant and antimicrobial activity than other varieties (Bangla, Sanchi, and Misti). The cytotoxicity activity in FBL extract in respect of 50% cell viability in human epithelial cell (HeLa), human neuroblastoma clonal cell (SH-SY5Y), and mesenchymal stem cells (MSCs) were 0.19 to 0.32 mg/mL, 0.41 to 0.54 mg/mL, and 0.46 to 0.96 mg/mL, respectively. On the other hand, the cytotoxicity activity of 50% cell viability showed 3.14 to 3.21 μg/mL, 5.81 to 6.06 μg/mL, 28.47 to 32.13 μg/mL in HeLa, SH-SY5Y, and MSCs cells; separately for hydroxychavicol and eugenol. In cancer cell lines, an increased cell death rate was observed with an increase in the betel leaf extract concentration but normal cells were unaffected. These results suggested that *Piper betle* L. leaves could be a potentially good source of bioactive anticancer agents.

In a nutshell, betel leaves were found to be good sources of essential elements, rich sources of volatile and non-volatile organic compounds, with several biochemical activities. Further research studies are needed on the betel leaf to isolate and characterize the identified major volatile and non-volatile organic compounds. The present study will be important for its more valuable and target-specific applications in controlling several diseases related to humans and other animals.

요약

Piper betle L. 잎은 전 세계적으로 저작, 구강청결 및 약용 목적으로 널리 사용된다. 이러한 *Piper betel* L. 잎은 방글라데시에서 품질, 안전성 및 효능에 대한 데이터가 부족한 실정이다. 본 연구는 5 종류의 *Piper betle* L. 잎 (FBL) 인 Bangla, Sanchi, Misti, Khasia 및 BARI Pann3 의 품종별 화학적 (무기 원소 : 다량, 미량, 초미량, 독성원소, 휘발성 및 비휘발성 유기화합물) 및 생화학적 (항산화, 항균 및 세포 독성) 특성 평가를 목표로 하였다.

유도결합플라즈마 분광분석기 (ICP-OES) 및 유도결합플라즈마 질량분석기 (ICP-MS) 을 이용한 5종류의 *Piper betle L.* 의 무기원소 분석결과 (다량 무기원소 : K> Ca> Mg> P> Na; 미량 무기원소 : Mn> Fe> Sr> Ba> Zn> Cu> Cr> Ni; 초미량 무기원소 : Ga> Li> V> Cs> Se> U> Ba; 독성원소 : Pb> As> Cd> Tl) 으로 확인하였다. 영양학적으로 중요한 무기원소 중 분석한 모든 betel 잎에서 K (3.99-5.86 g/kg), Ca (2.64-2.80 g/kg) 및 Mg (1.26-1.33 g/kg) 으로 확인되었다. 반면, 세계 보건기구 (WHO) / 식량 농업기구 (FAO) 에서 지정한 한계 기준 Mn (21.94-25.09 mg/kg) 및 Pb (129.36-965.91 μg/kg) 를 제외한 모든 무기원소에서 안전한 수치로 확인되었다.

동시증류추출 및 가스크로마토그래피 질량분석법 (SDE/GC-MS) 을 사용하여 FBL의 휘발성 유기화합물 분석을 하였다. Betel 잎 중 Misti 종에서 가장 많은양의 휘발성 유기화합물 (13958.90 mg/kg) 이 확인되었으며, BARI Paan3 (11684.10 mg/kg), Khasia (11109.70 mg/kg), Sanchi (6958.51 mg/kg) 및 Bangla (4346.91 mg/kg) 순으로 확인되었다.

휘발성 유기성분 분석을 통하여 총 101종의 화합물을 확인하였다. 이 중 42종의 화합물은 공통으로 발견되었으며, 현재까지 연구된 타 문헌과 비교 시 본 연구를 통하여 betel 잎에서 새로운 50종의 휘발성 화합물을 확인하였다. Eugenol 은 모든 betel 잎에서 주요 화합물로 확인되었으며, 기타 주요 화합물은 β-caryophyllene, γ-muurolene, valencene, eucalyptol, chavicol 및 caryophyllene oxide 이었다.

비휘발성 유기화합물 스크리닝 테스트를 통해 FBL 의 phenol, flavonoids, terpene, steroids, phytosterols 및 saponin을 확인하였다. 또한, CCC 설계(central composite circumscribed)와 RSM (response surface methodology) 을 사용하여 betel 잎에서 초음파 추출을 통한 최적의 총 페놀 함량을 추출하기 위한 방법을 마련하였다. 최종 사용된 용매로 ethanol : acetic acid : water (70 : 5: 25 v/v) 를 선정하였으며, 최적의 추출 조건은 추출 시간 90분, 추출 온도 75℃; 고체/액체 비율 1:15.41로 지정하였다. 확립한 최적의 초음파 추출 조건에 의해 FBL를 추출하였으며, 총 페놀 및 플라보노이드 함량은 각각 110.51-322.8 mg GAE/g DW 및 46.79-57.09 mg QE/g DW로 확인되었다. 이후 주요 페놀 화합물인 hydroxychavicol 를 액체크로마토그래피-다이오드 검출기 (HPLC-DAD) 를 이용하여 FBL의 14.19 mg/g – 38.19 mg/g 으로 정량하였다.

2,2-Diphenyl-1-pycrilhydrazil (DPPH) 및 2,2'-Azino- (bis 3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) 분석 시 FBL 추출물에서 IC_{50} 값의 각각 황산화 활성 0.17-0.43 mg/mL, 0.04-0.11 mg/mL 으로 확인되었다. DPPH 및 ABTS 의 주요 페놀 화합물 IC_{50} 값은 hydroxychavicol 및 eugenol 에 대해 각각 10.88-14.47 μg/mL 및 7.66-18.00 μg/mL이었다. 또한, Betle 잎 추출물은 *S. typhimurium, P. aeruginosa, E. coli, A. faecalis,* and MRSA

박테리아에 대하여 우수한 억제 특성을 나타냈다. Khasis 와 BARI Paan 3 은 다른 품종 (Bangla, Sanchi, 및 Misti) 보다 높은 항산화 및 항균 활성을 보였다. 인간 상피 세포 (HeLa), 인간 신경 모세포종 클론세포 (SH-SY5Y) 및 중간엽줄기세포 (MSCs) 의 IC_{50} 값에 따른 FBL 추출물의 세포독성 활성은 각각 0.19-0.32 mg/mL, 0.41-0.54 mg/mL, 0.46-0.96 mg/mL 으로 확인되었다. 반면, hydroxychavicol 및 eugenol 의 세포독성활성 (IC_{50}) 은 HeLa, SH-SY5Y 및 MSCs 세포에서 각각 3.14-3.21 μg/mL, 5.81-6.06 μg/mL, 28.47-32.13 μg/mL 을 나타냈다. 암세포중에서 betle 잎의 추출물 농도가 증가하면 암세포의 사멸률이 증가하였으며, 정상세포는 영향을 받지 않았다. 본 연구를 통하여 *Piper* betle L. 잎이 잠재적으로 생체 활성 항암제 공급원으로 사용될 수 있음을 기대볼 수 있다.

Betle 잎은 여러 가지 생화학적 효능과 함께 필수영양소의 좋은 공급원, 휘발성 및 비휘발성 유기화합물의 공급원으로 확인되었다. Betle 잎에 대한 추가적 연구로 확인된 주요 휘발성 및 비휘발성 유기화합물을 분리 및 특성화하고자한다. 또한, 현재의 연구는 건강 및 기타 동물과 관련된 여러 질병을 제어하는데 있어 가치 있고 특정 분야에 중요할 것으로 판단된다.

CHAPTER I

Analysis of major, minor, trace, and toxic elements five varieties of *Piper betle* L. leaves (FBL) from Bangladesh

1.1. Introduction

Piper betle L. leaves are delicious, evergreen creeper, aesthetic, and have ritual significance in various social and religious occasions. Betel leaf, areca nut, lime, and areca catechu are considered as "family members" with deep respect in the culture of some South Asian counties, especially in Bangladesh, India, Pakistan, and etc (Karak et al., 2016). However, it is believed that the betel leaves are native to central and eastern Malaysia, and it was first cultivated about 2500 years ago in Malaysia and tropical Asia. After that, it arrived in Madagascar and East Africa, and it was also introduced to the West Indies. South-East Asia was mentioned as a region of betel users in written sources from the Tang dynasty (AD 618-907). When the first Europeans arrived in the 15th century, betel chewing was popular in South India and South China (Kumar, 1999). Betel leaves are consumed in different forms by more than 600 million people worldwide, and it ranks at third position, surpassed only by tea and coffee (Pradhan et al., 2014). These leaves have different names in various countries worldwide, where "Paan" is the most famous name in Bangladesh, Nepal, Pakistan, India, and different language local names are given in Table 1.1 (Basak and Guha, 2015).

Betel leaves are a shade-loving, perennial, semi-woody climber that grows well in humid tropical climates. The plant has long-stalked, deep green heart-shaped, smooth, shining leaves. These leaves have an aromatic flavor and a heavy pungent odor with economically and medicinally importance. The fresh leaves around moisture content (85 - 90%), protein (3 - 3.5%), fat (0.4 - 1%), minerals (2.3 - 3.3%), fiber (2.3%), and carbohydrates (0.5-6.1%) with vitamin B and vitamin C, as well as being helpful in the digestive process (Shah et al., 2016; Arawwawala

Table 1.1. The common name of *Piper betle* L. leaves in different region worldwide

No	Language	Vernacular Name
1.	Arabic, Persian	Tambol, Tambool
2.	Bangali	Paan
3.	Burmese	Kun
4.	Chamorro, Austronesian	Pupulu
5.	Dhivehi, Maldivian	Bileiy
6.	English	Betel, Betel pepper, Betel vine
7.	Gujarati	Nagarbael
8.	Hindi	Paan
9.	Jakun	Kerekap, Kenayek
10.	Javanese	Sirih, Suruh, Bodeh
11.	Kalinga	Gaweud
12.	Khmer, Cambodian language	Maluu
13.	Laos	Pu
14.	Malaysia	Sirih, Sirih carang, Sirih kerakap
15.	Malaysian, Indonesian	Daun sirih
16.	Mon, Austroasiatic language	Plu
17.	Sakil	Jerak
18.	Semang	Serasa, Cabe
19.	Sinhalese	Bulath
20.	Tagalog, Austronesian language	Ikmo
21.	Tamil	Vetrilai
22.	Telugu	Nagballi, Tamalapaku
23.	Thai	Pelu
24.	Tokodede, language of East Timor	Maluu
25.	Vietnameses	Trau

et al., 2014). It also acts as a tasty appetizer and helps to remove oral itching and bad odors. The young fresh leaves contain more essential oil than old leaves and it contains 0.08 to 0.3% and

various bioactive compounds which are used as raw materials in pharmaceutical and cosmetic industries (Islam et al., 2020[a]). Some studies have confirmed over 100 varieties of betel leaf worldwide (Guha, 2006). On the basis of chemical constituents, some varieties are recognized, such as, Bangla, Kapoori, Misti (Meetha), Sanchi, Desawari, Khasia, BARI Paan 3 (Islam et al., 2020[a]; Rawat et al., 1989).

In Bangladesh, betel leaves are widely cultivated in Sylhet, Moulivibazar, Jessore, Kustia, Khulna, Satkhira, Bagerhat, Narail, Bhola, Faridpur, Barisal, Rajshahi, Rangpur, Gaibanda, pabna, Cox's Bazar and greater Chittagong district. Betel leaf cultivation process can be divided into two groups, the plain land betel leaves (boroj paan) and tree-oriented betel leaf (gach paan). In greater Sylhet districts, Khasia people cultivates tree betel leaf or gach paan, locally calls it Khasia paan. Betel leaf cultivation has been reported on about 12,660 - 18,247 hectares of farmland, with an annual production of 0.06 - 0.10 million tons (Islam et al., 2015). Around 60-70% of people usually consume betel leaves frequently in Bangladesh (Hossain et al., 2017). It is a very important economic crop, which exports to Saudia Arabia, United Arab Emirates, Middle East, The United Kingdom, Pakistan, India and a few other African nations. Betel leaves usages are increasing in many sectors worldwide due to their phytochemical importance.

1.1.1. Importance of mineral elemental analysis in *Piper betle* L. leaves

Elements are inorganic nutrients, and it is very necessary to maintain in human, animal, and plant nutrition. Normally less than 1 to 2500 mg/day is needed for the human body, depending on the mineral types, mainly compared with other nutrients, such as carbohydrates and lipids (Soetan et al., 2010). At least 23 mineral elements are required to function in our body, and there are many methods established to determine the nutritional status of mineral elements (Quintaes et al., 2015). As with essential food nutrients, vitamins and mineral requirements vary with animal species. A mineral element is considered essential when deficient ingestion results

in harm with physiological levels of this specific element repair or prevents it. The maximum or minimum amount of mineral elements are produced adverse health effects, which can vary among different mineral elements (Belitz et al., 2009). So, betel leaf elemental status understanding is very important. There are limited information on the mineral elements (major, minor, trace, and toxic) content of betel leaves in Bangladesh.

1.1.1.1. Major mineral elements

The major mineral elements such as potassium (K), magnesium (Mg), calcium (Ca), phosphorous (P), and sodium (Na) are essential for the human body and it is also known as main nutrients. Generally, more than 100 mg/day is required for the human body (Karadas and Kara, 2012; Gonzalvez et al., 2008; Fraga, 2005). Sodium maintains osmotic pressure and acid-base balance for the human body. It is also essential to an active enzyme such as amylase (Belitz et al., 2009; Page and Di Cera 2006). Calcium is very important for maintaining and building bones, blood clotting, muscle contraction (Pravina et al., 2013; Belitz et al., 2009). Potassium plays an important role in the normal functioning of the cell. It regulates the heartbeat as well as ensures the proper function of the muscles and nerves, and it is also a vital role plays for synthesizing protein and metabolizing carbohydrates (Belitz et al., 2009). Magnesium is a life-supporting element and it is also required for insulin activity for the prevention of type II diabetes (Belitz et al., 2009; Huerta et al., 2005). Likewise, phosphorous plays a very significant role in metabolism and is essential for bone development along with other aforementioned minerals (Belitz et al., 2009). Good analytical techniques are essential to monitoring the concentration of these major mineral elements in foods, for example, betel leaf. The deficiency or increase of mineral element concentration causes serious disturbances in the whole physiological system (Chaitanya and Sahu, 2020; Belitz et al., 2009; Page and Di Cera, 2006).

1.1.1.2. Minor mineral elements

The minor elements such as chromium (Cr), nickel (Ni), manganese (Mn), copper (Cu), barium (Ba), iron (Fe), zinc (Zn), and strontium (Sr) are essential for the human body. Generally, less than 100 mg/day is required for the human body (Karadas and Kara, 2012). The concentration of minor elements is recommended in the human body by World Health Organization (WHO) and Food and Nutritional Board (FAO), institute of medicine, DC, USA (FNB 2005; WHO 1996; FAO and WHO 1983). Some minor elements such as manganese, chromium, zinc, and copper are considered essential elements that belong to the group of major elements (Karadaş and Kara 2012). In biological systems, minor elements are mostly conjugated to proteins forming as metalloproteins, or other smaller molecules, in the form of phytates, phosphates, polyphenols, and other chelating compounds. Minor elements like chromium, copper, and nickel are the essential components of molecular biological structures, but they can play a toxic nature when that concentration over those required for their biological functions (Karadas and Kara, 2012; Page and Di Cera, 2006).

1.1.1.3. Trace mineral elements

The trace elements are needed for the human body, such as lithium (Li), beryllium (Be), vanadium (V), cobalt (Co), gallium (Ga), cesium (Cs), selenium (Se), and uranium (U). These trace elements are observed and categorized in various food samples (Tokalioğlu, 2012; Singh and Garg, 2006). According to Khan et al. (2014) state that the above describe trace elements are nontoxic trace elements. Cobalt, selenium, and vanadium are considered essential or probable essential trace elements, respectively (WHO, 1996). In comparison, all others are not known for any noticeable nutritional significance. Selenium plays an important role in catalyzing oxidation-reduction reactions. The dietary reference intake (DRI) and tolerable upper level (TUL) for Se were 45 and 400 µg/day specified by JECFA and Food Nutrition Board, USA, respectively (FNB, 2005; FAO and WHO, 1983). In biological systems, trace elements along with other minor

elements are usually present as phosphates, phytates, polyphenols, and metalloproteins or other chelating compounds. These have a critical structural function or enzymatic may be involved in transportations in our body (Fraga, 2005).

1.1.1.4. Toxic mineral elements

Toxic elements such as arsenic (As), lead (Pb), cadmium (Cd), and thallium (TI) are well-known toxic elements. Toxic elements are very important to be considered for food analysis. Lead is a wide speared environmental hazard and it has neurotoxic effects. Lead is a major public health concern worldwide (Verstraeten et al., 2008). Similarly, research studies have indicated that lead is abundant in food at a concentration of several hundred µg/g (Islam et al., 2020[b]; Muhib et al., 2016; Naser et al., 2009). Therefore, it is essential to know the lead concentrations and realize the toxicity level in betel leaves from Bangladesh.

1.1.2. Analysis of mineral elements

Recently, there are many analytical techniques established for the determination of major, minor, trace, and toxic elements, namely: instrumental neutron activation analysis (Singh and Garg, 2006), different pulse anodic stripping voltammetric technique (Silveira et al., 2013), capillary zone spectrometry (Hakkarainen and Matilainen, 2009), stripping potentiometry (Suturović et al., 2019), flame atomic absorption spectrometry (Kondyli et al., 2007), flow injection spectrometric methods (Nogueira et al., 1998), inductively coupled plasma optical emission spectrometry (ICP-OES) (Kira and Maihara, 2007), and inductively coupled plasma mass spectrometry (ICP-MS) (Khan et al., 2014[a]). ICP-OES technique was successfully applied for the analysis of major nutrition elements in dairy products by Khan et al., 2014[b]. ICP-MS technique has been widely used for the analysis of minor, trace, and toxic elements in foods (Shchukin et al., 2020; Khan et al., 2014[a]) with acceptable results.

1.1.2.1. Microwave digestion system

Microwave digestion is an established technique used by analytical scientists for 30 years. It dissolved mineral elements in the organic molecule's presence before analyzing inductively coupled plasma mass spectrometry and inductively coupled plasma-optical emission spectrometry. This technique is usually accomplished by exposing a sample to a strong acid in a closed vessel and raising the pressure and temperature through microwave irradiation (Khan et al., 2014[b]). This increase in temperature and pressure of the low pH sample medium increases both the speed of thermal decomposition of the sample and the solubility of mineral elements in the solution. Once these mineral elements are in solution, it is possible to quantify the sample through different elemental analysis techniques.

1.1.2.2. Inductively coupled plasma-optical emission spectrometry

Inductively coupled plasma-mass spectrometry (ICP-OES) is an analytical technique where the composition of mineral elements in the sample (mostly water dissolved) can be determined using plasma and a spectrometer. It is a type of emission spectroscopy that uses the inductively coupled plasma to produce excited atoms and ions that emit electromagnetic radiation at wavelengths characteristics of a particular element. The plasma is a high-temperature source ionized by argon gas. The plasma is sustained and maintained by inductively coupled from cooled electrical coils at megahertz frequencies. The source of temperature ranges from 6000 to 10000 K. The intensity of the emission from various wavelengths of light is proportional to the concentrations of the sample elements (Khan et al., 2014[c]).

1.1.2.3. Inductively coupled plasma-mass spectrometry

Inductively coupled plasma-mass spectrometry (ICP-MS) is an elemental analysis powerful technique for trace multi-element and isotopic analysis. This technique can detect most

of the elements in the periodic table at mg/kg to ng/kg. The inductively coupled plasma (ICP) is an ionization source that completely decomposes a sample into its constituent elements and transforms these elements into ions, which are detected. It can detect different isotopes of the same element, which makes it a versatile tool in isotopic labeling (Nardi et al., 2009).

1.1.4. Justification of the study

Piper betle L. is important to consider both inorganic and organic components to determine the plant's therapeutic effectiveness. The consumption of medicinal plants does not preclude the ingestion of inorganic constituents for organic compounds. Medicinal plants can absorb and store heavy metals from the atmosphere. Polluted medicinal plants can lead to adverse health effects and metal toxicity. Good analytical techniques are needed regularly to understand the concentration levels of major, minor, trace, and toxic elements in food samples. The whole physiological system may cause serious disturbances when important elements have any deficiency or increase apart from their critical limits in food samples (Belitz et al., 2009). Recently, there are many analytical methods used for elemental analysis in foods. ICP-OES and ICP-MS are the most popular methods due to their well-known advantage of selectivity, sensitivity, and multi-element analysis capability (Choi et al., 2014; Nogueira et al., 1998). In addition, microwave digestion method for food sample preparation with satisfactory results (Nho et al., 2016; Khan et al., 2013). To the best of the author's knowledge, there are no comprehensive studies for well-known major, minor, trace, and toxic element levels in FBL, widely consumed in Bangladesh and worldwide. This work aims to analyze the concentrations of 25 elements in FBL from Bangladesh. The selected samples were prepared by microwave-assisted acid digestion and twenty-five elements were analyzed by ICP-OES and ICP-MS technique. The applied techniques and the analytical performance were validated by quality assurance parameters such as limits of detection and quantification, linearity, precision, and spiking recovery experiments. The accuracy is analyzed by certified reference material (NIST CRM-

1570a) named Spinach leaves. WHO/FAO specified the toxic metal critical levels to compare the analyzed toxic elements results. Principal component analysis (PCA) and hierarchical cluster analysis (HCA) were also performed to determine the presence of possible similarity and classification patterns of samples via elemental concentration.

1.2. Materials and Methods

1.2.1. Samples collection

Fresh betel leaves of five selected varieties were collected in triplicate from Bangladesh. Out of these, one betel leaf sample BARI Paan 3 was collected from Spice Research Institute, Bogra, Bangladesh, while the other four common local varieties were collected from specific shade gardens with traditional farming systems, as shown in Table 1.2. For each variety, fresh, healthy, green, and mature betel leaves with different sizes were collected from more than 200 plants in February 2019. The samples were verified by the Bangladeshi regional agricultural extension field officer. After collecting, the betel leaves were washed thoroughly in clean water. Finally, it was washed with distilled water. Then the clean leaves were dried at room temperature for approximately 14 days. All dry leaves were ground into powder using a blender (MR 350CA, Braun, Spain) and stored at 4 °C before the experiment.

Table 1.2. Sample collection information in FBL from Bangladesh

Symbol	Scientific name	Local name	Origin	Latitude and Longitude
A	*Piper betle* L. var. Bangla	Bangla Paan	Durgapur, Rajshahi, Bangladesh	24˚29´27´´ N and 88˚42´19´´ E
B	*Piper betle* L. var. Sanchi	Sanchi Paan	Durgapur, Rajshahi, Bangladesh	24˚31´18´´ N and 88˚43´27´´ E
C	*Piper betle* L. var. Misti	Misti Paan	Moheskhali, Chittagong, Bangladesh	21˚31´10´´ N and 91˚57´52´´ E
D	*Piper betle* L. var. Khasia	Khasia Paan	Sirimongol, Sylhet, Bangladesh	24˚09´22´´ N and 91˚44´37´´ E
E	*Piper betle* L. var. BARI Paan 3	BARI Paan 3	Spice research center, Sibgonj, Bogra, Bangladesh	24˚58´42´´ N and 89˚20´15´´ E

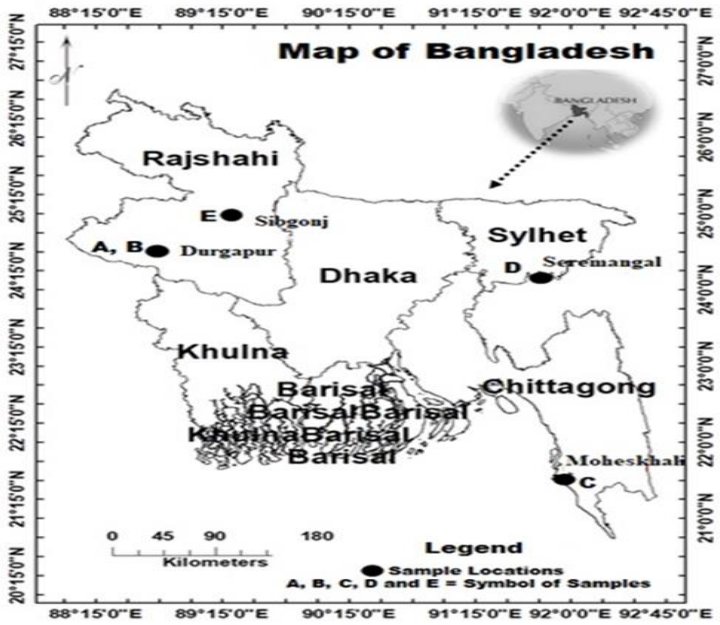

Figure 1.1. Samples collection locations map in FBL from Bangladesh.

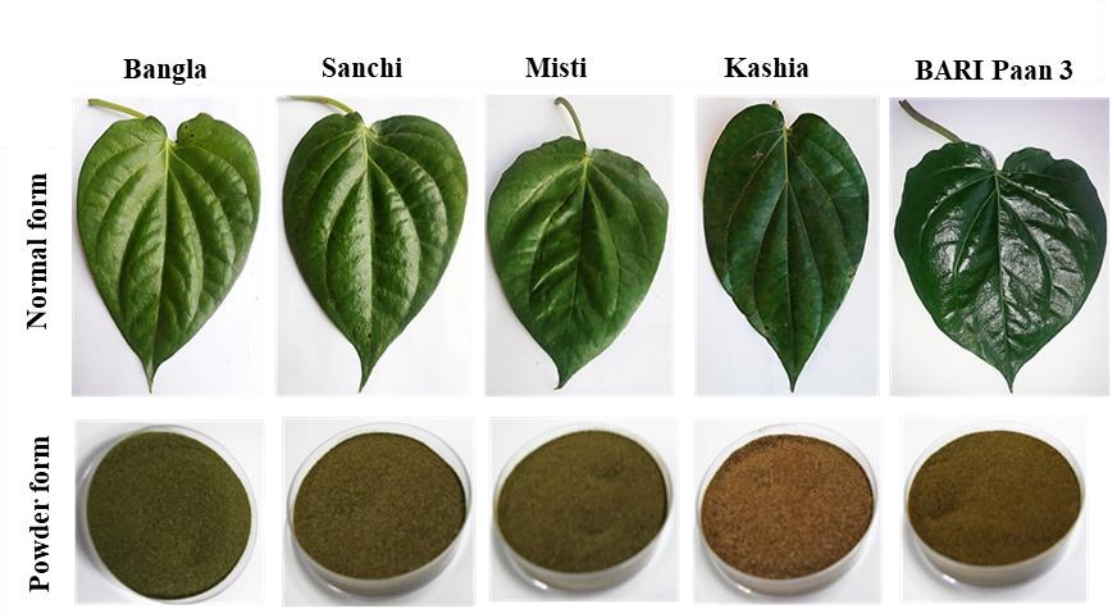

Figure 1.2. Picture of FBL (fresh and powder form) from Bangladesh.

1.2.2. Reagent and chemicals

All analytical-grade chemicals or reagents were used in this analysis, such as ultra-pure deionized water (>18.0 $M\Omega cm^{-1}$), hydrogen peroxide (H_2O_2), nitric acid (HNO_3). The multi-element standards (100 and 10 mg L^{-1}) and standard reference material ($SRM\text{-}1570_a$, Spinach leaves) were purchased from the National Institute of Science and Technology (NIST) (Gaithersburg, MD, USA), individually. All the plastic and glassware were washed thoroughly with detergent and clean water, then socked in 10 % HNO_3 solution overnight and it was rinsed several times with deionized water.

1.2.3. Analytical apparatus

The elemental analysis of *Piper betle* L. related apparatus details are shown in Table 1.3.

Table 1.3. Elemental analysis related apparatus list

S. N.	Apparatus name	Description
i.	Microwave digestion	TOPwave, Analytik Jena, Germany
ii.	ICP-OES	Optima8000, Perkin Elmer, CT, USA
iii.	ICP-MS	300D, Perkin Elmer Sciez, CT, USA

1.2.4. Samples preparation and digestion of mineral elements

Firstly, betel leaves samples were prepared using nitric acid-assisted microwave digestion. Briefly, 0.5 g of the dried powder sample (in triplicate) was digested and it was decomposed using 7.0 mL of 70% HNO_3 and 1.0 mL of 30% H_2O_2 in microwave polytetrafluoroethylene digestion vessels. The microwave digestion system was operated at a power of 0 W for cooling purposes. The temperature program was set as 80˚C for 5 minutes, 50˚C for 15 minutes, 190˚C for 5 minutes, 190˚C for 20 minutes. After completing the combustion and decomposition processes, the digestion vessel content was transferred to 50 mL tubes. To obtain 30.0 g weight, the combustion samples were diluted with deionized water, filtered, and then subjected to elemental analysis. The major elements: calcium (Ca), potassium (K), magnesium (Mg), sodium (Na); and phosphorus (P), as well as minor minerals: chromium (Cr), manganese (Mn), nickel (Ni), copper (Cu), strontium (Sr), barium (Ba), iron (Fe), and zinc (Zn); trace elements: lithium (Li), beryllium (Be), vanadium (V), cobalt (Co), gallium (Ga), cesium (Cs), selenium (Se), and uranium (U); and toxic elements: arsenic (As), cadmium (Cd), thallium (TI) and lead (Pb), were analyzed by ICP-OES and ICP-MS, respectively. The optimized ICP-OES and ICP-MS instrumental parameters and operating conditions are described

in Table 1.4. Data quantification and drift correction of all elements was achieved by analyzing the multi-element standards at the intervals of every ten sample analyses.

Table 1.4. Optimized ICP-OES and ICP-MS instrumental parameters and operating conditions

ICP-OES	:	Optima8000, Perkin Elmer
Nebulizer	:	Sea spray
Spray chamber	:	Cyclonic
RF generator (MHz)	:	27.12
Argon gas flow rates	:	
Plasma (L min^{-1})	:	16
Auxiliary (L min^{-1})	:	1.5
Nebulizer (L min^{-1})	:	0.94
Plasma viewing	:	Axial
Elemental wavelengths	:	Major elements: K (766.490), Ca (317.933), Mg (285.213), P (213.617) and Na (589.592)
ICP-MS	:	300D, Perkin Elmer
Nebulizer	:	Meinhard
Spray chamber	:	Cyclonic
RF power (kW)	:	1.35
Argon gas flow rates	:	
Plasma (L min^{-1})	:	16
Sample depth (mm)	:	6.0-8.0
Auxiliary (L min^{-1})	:	1-1.3
Nebulizer (L min^{-1})	:	1.0-1.07
Element isotopes		Minor, trace and toxic elements: Li^{7}, Be^{9}, V^{51}, Cr^{52}, Mn^{55}, Ni^{60}, Cu^{63}, Sr^{88}, Co^{59}, Ba^{138}, Fe^{58} ,Zn^{66}, Ga^{69}, Se^{82}, U^{238}, Cs^{133}, AsO^{91}, Pb^{208}, Cd^{111} and TI^{205}

1.2.5. Calibration procedure

The calibration technique is very important for the quantitative analysis of the sample. A multi-element standard solution was prepared by 19.6 % (W/W) HNO_3. The calibration curves for all the analytes were made on eight different concentrations. The limit of detection (LOD) of the corresponding element in the samples was within a linear range of the calibration curve. The calibration standards were analyzed at regular intervals during analysis as samples to monitor the instrumental drift. Ultrapure deionized water used frequently as a blank in analyzed samples to check for any loss or cross-contamination. Any

slight instrumental drift was taken into account to avoid any possible error. Every measurement was carried out using the full quantitative analysis mode. The absence of polyatomic interferences was checked by measuring several isotopes of the elements to find out the most abundant isotope with the least interference. For example, in the case of selenium, three isotopes (^{77}Se, ^{78}Se, and ^{82}Se) were checked and found ^{82}Se with no interference and selected for measurement in the subject betel leaves.

1.2.6. Quality assurance

The analytical method followed for the determination of major, minor, trace, and toxic elements in betel leaves were validated by measuring several quality parameters, including sensitivity, linearity, precision, accuracy, and spike recovery. The instrument sensitivity was established through the determination detection of limits for all elements studied. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated with three and ten times the standard deviation of the blank divided by the analytical curve slope. Linearity was established by preparing all analyte element's calibration curves using a non-weighted least-squares linear regression analysis method. All calibration curves were prepared with eight standard solutions, including the blank. These were prepared in such a way that the concentrations of all analytes elements in the samples were within the linear range of calibration curves and above the established lower linearity limit (Shchukin et al., 2020). Precision is described as the degree of variability given by the expression of results, not taking into account the influence of the samples (sample variability). Khan et al., 2014[b] evaluated precision by using a relative standard deviation of 10 repeated determinations of one sample. Following this method, the percent coefficient of variation (CV%) was obtained for all analyte elements. The accuracy of the method was checked by analyzing the SRM-1570a, Spinach leaves, for the determination of the major elements: Ca, K, Mg, Na, and P as well as minor minerals: Cr, Mn, Ni, Cu, Sr, Ba, Fe; trace elements: Li, Be, V, Co, Ga, Se, Cs, and toxic elements: As, Cd, TI, and Pb were

analyzed using ICP-OES and ICP-MS, respectively. The analytical quality control for all 25 selected elements was also verified via recovery experiments by spiking at two selected concentrations of 1000 µg/kg and 100 µg/kg (Khan et al., 2014[a]).

1.2.7. Statistical analysis

The Statistical Package for the Social Science (SPSS), version 23 (IBM, New York, USA) was used for the statistical analysis. The main data was generated from the three batches of samples. The significant differences between their mean values were analyzed to use analysis of variance (ANOVA) followed by Duncan's multiple range tests at a 5% significance level. The final report was shown as a mean ± standard deviation of triplicate measurements. Past statistical software (version 3.25) was used for principal component analysis (PCA) and hierarchical cluster analysis (HCA). It helped to explore the relationships between the analyzed elements and the samples.

1.3. Results and Discussions

1.3.1. Validation of analytical methods

The determination of important quality parameters, including correlation coefficient (R^2), limit of detection (LOD), limit of quantification (LOQ), precision (RSD), and spike recovery, are shown in Table 1.5. This analytical method was followed by the Association of Official Analytical Chemists (AOAC) for analysis (Khan et al., 2014[b], Khan et al., 2013; AOAC, 2012). The correlation coefficient was calculated from the calibration curves with values from 0.99751 (Mg) to 0.99992 (Mn) (Table 1.5). The LOD and LOQ allowed the determination to the required level. The recovery values means of all the analyte elements were found to be within the interval of confidence ($p > 0.05$) calculated for the certified values of standard reference material (SRM). Similarly, the recovery percentages for all 25 analytes were in the range of

Table 1.5. Correlation of determination (R^2), limit of detection (LOD), limit of quantification (LOQ), precision (RSD), and spike recovery data of the ICP-OES for quantification of nutritional elements

Instrument	Element	R^2	LOD	LOQ	RSD (%)	Recovery (%)
			Major (mg/kg)			
ICP-OES	Ca	0.99789	0.166	0.548	5.899	93.817
	K	0.99873	0.149	0.493	5.125	97.168
	Mg	0.99751	0.117	0.386	4.220	92.327
	Na	0.99875	0.072	0.238	2.396	100.219
	P	0.99872	0.179	0.592	6.620	90.359
			Minor (µg/kg)			
ICP-MS	Cr	0.99968	0.317	1.047	3.709	95.036
	Mn	0.99992	0.197	0.649	2.016	108.446
	Ni	0.99939	0.249	0.821	2.580	107.164
	Cu	0.99923	0.192	0.635	2.213	96.612
	Sr	0.99863	1.462	4.826	6.975	95.716
	Ba	0.99985	0.363	1.199	3.743	107.821
	Fe	0.99923	0.035	0.116	1.155	101.765
	Zn	0.99987	0.050	0.164	1.633	101.546
			Trace (µg/kg)			
	Li	0.99829	0.362	1.194	3.735	107.680
	Be	0.99799	0.206	0.679	2.159	105.836
	V	0.99918	0.710	2.341	6.798	115.973
	Co	0.99951	0.171	0.564	1.751	108.564
	Ga	0.99825	3.364	11.100	2.745	114.140
	Se	0.99977	0.242	0.799	2.827	95.219
	Cs	0.99925	0.127	0.419	1.240	113.790
	U	0.99961	0.504	1.664	5.177	108.202
			Toxic (µg/kg)			
	As	0.99925	0.149	0.490	1.506	109.640
	Cd	0.99957	0.130	0.430	1.335	108.498
	Tl	0.99915	0.149	0.491	1.481	111.497
	Pb	0.99951	0.331	1.091	3.755	97.791

92.327% to 109.640%, as represented in Table 1.6. Analyzing the spinach leaves (NIST-1570a) and their recoveries were obtained 92.041 to 101.919% for ICP-OES and ICP- MS analysis,

respectively (Table 1.6). All the estimated quality parameter values were fulfilled the required criteria according to the Association of Official Analytical Chemists (AOAC).

Table 1.6. Analysis of certified reference material (NIST-1570a), spinach leaves by ICP-OES (n = 5)

Unit: mg/kg

Element	Certified value	Observed value	Recovery (%)
Ca	15260 ± 66	14820 ± 38	97.116
K	29000 ± 260	27752 ± 114	95.969
Mg	8910 ± 90	9081 ± 10	101.919
Na	18210 ± 230	17790 ± 80	97.693
P	5187 ± 67	4980 ± 50	96.009
Cu	12.20 ± 0.6	11.40 ± 0.25	93.442
Mn	76.00 ± 1.2	72.80 ± 0.08	95.789
Ni	2.14 ± 0.058	2.01 ± 0.03	93.925
Zn	82.00 ± 3.9	80.2 ± 0.58	97.804
As	0.068 ± 0.012	0.067 ± 0.013	98.529
Cd	2.89 ± 0.07	2.66 ± 0.05	92.041
Co	0.39 ± 0.05	0.38 ± 0.07	97.435
Se	0.117 ± 0.0009	0.115 ± 0.003	98.290
V	0.57 ± 0.03	0.55 ± 0.04	96.491

1.3.2. Major mineral elements in FBL

The analyzed major elements concentration was roughly closed among the FBL from Bangladesh. Rehan et al. (2018) reported a similar trend of major element concentration in Pakistani betel leaves. The concentration of K (3.99 – 5.86 g/kg), Ca (2.64 – 2.80 g/kg), and Mg (1.26 – 1.33 g/kg), were the most abundant nutritional elements in betel leaves, followed by P (0.28 – 0.31 g/kg), Na (0.12 – 0.30 g/kg) (Table 1.7). Comparing all major elements amount was lower than reported betel leaves studies on dry weight basis calculation in the literature (Nho et al., 2016). The analyzed major elements are very well-known nutritional elements. These elements are needed for various physiological actions in the body, such as blood clotting, osmotic pressure, acid-base balance, muscle contraction, bone development, enzymatic activities, and

hemoglobin synthesis (Belitz et al., 2009). The ratio of Na/K in any food item is an important factor; too much Na and less K consumption contribute to a high prevalence of hypertension (Tanase et al., 2011). The Na/K ratio in our body is very important to control high blood pressure and the ratio should be less than one (Akubugwo et al., 2007). In our study, betel leaves Na/K ratio less than one (0.035), which indicates that the consumption of betel leaves is helpful for humans and might control the high blood pressure in our body.

Table 1.7. Determination of the major element in FBL from Bangladesh

Unit: g/kg

Betel varieties	Ca	K	Mg	Na	P
Bangla	$2.64^{b} \pm 0.17$	$5.86^{ab} \pm 2.00$	$1.26^{b} \pm 0.02$	$0.16^{a} \pm 0.20$	$0.28^{b} \pm 0.02$
Sanchi	$2.78^{b} \pm 0.18$	$4.73^{a} \pm 0.69$	$1.32^{b} \pm 0.13$	$0.13^{a} \pm 0.15$	$0.30^{b} \pm 0.02$
Misti	$2.76^{b} \pm 0.10$	$4.30^{bc} \pm 0.77$	$1.20^{b} \pm 0.89$	$0.12^{a} \pm 0.44$	$0.29^{b} \pm 0.04$
Khasia	$2.80^{a} \pm 0.14$	$3.99^{c} \pm 0.59$	$1.33^{a} \pm 0.06$	$0.12^{a} \pm 0.28$	$0.31^{a} \pm 0.01$
BARI Paan 3	$2.73^{c} \pm 0.14$	$4.83^{c} \pm 0.50$	$1.27^{b} \pm 0.12$	$0.30^{a} \pm 0.26$	$0.29^{c} \pm 0.02$
Average	2.76	4.74	1.29	0.16	0.30

Values are mean ± standard deviation of three (n = 3) measurements. The superscript letters (a-c) in a row for nutritional elements present significantly different values in five varieties of betel leaves ($p < 0.05$) by Duncan's multiple range tests

1.3.3. Minor mineral elements in FBL

In this study, the minor elements were categorized as those having an average amount of more than 0.50 mg/kg for the analysis in 5 varieties of betel leaf. These eight minor elements were included, namely Cr, Mn, Ni, Cu, Sr, Ba, Fe, and Zn. Among the minor elements concentrations, Mn (21.94 – 25.09 mg/kg), Fe (8.51 – 14.89 mg/kg), Sr (5.48 – 10.07 mg/kg), Ba (4.50 – 5.33 mg/kg), and Zn (3.17 – 3.88 mg/kg) were the highest in betel leaves, followed by Cu (1.44 – 1.84 mg/kg), Cr (0.75 – 1.22 mg/kg), and Ni (0.17 – 0.31 mg/kg) (Table 1.8). The World Health Organization (WHO) was classified essential elements (Cr, Cu, and Zn) and

probable essential elements (Mn and Ni) for the human body (Rehan et al., 2018; WHO, 2003). The permissible Mn limit in edible plants is 2 mg/kg and it was set by FAO/WHO (Tanase et al., 2011). This indicates that all analyzed sample manganese concentration was above this limit. The Mn concentration in the present study was lower than other Indian and Pakistan betel leaves studies (Rehan et al., 2018; Nema et al., 2014).

Table 1.8. Determination of the minor element in FBL from Bangladesh

Unit: mg/kg

Betel varieties	Cr	Mn	Ni	Cu	Sr	Ba	Fe	Zn
Bangla	1.22^{b} ± 0.20	24.11^{a} ± 0.28	0.17^{a} ± 0.09	1.84^{c} ± 0.19	5.48^{a} ± 0.19	5.33^{ab} ± 0.22	8.51^{a} ± 2.17	3.44^{b} ± 1.21
Sanchi	1.12^{a} ± 0.36	23.41^{a} ± 1.41	0.20^{a} ± 0.07	1.75^{a} ± 0.31	5.64^{a} ± 0.46	5.05^{a} ± 0.49	9.67^{b} ± 1.30	3.17^{b} ± 0.26
Misti	$0.97a^{b}$ ± 0.39	22.49^{b} ± 1.70	0.22^{b} ± 0.04	1.63^{c} ± 0.36	5.79^{d} ± 0.36	4.79^{b} ± 0.64	11.89^{b} ± 0.38	3.54^{a} ± 0.47
Khasia	0.75^{b} ± 0.02	21.94^{c} ± 0.77	0.20^{c} ± 0.01	1.44^{ab} ± 0.03	6.08^{b} ± 0.29	4.50^{c} ± 0.17	14.89^{a} ± 0.29	3.88^{ab} ± 0.3
BARI Paan 3	0.82^{a} ± 0.10	25.09^{d} ± 5.39	0.31^{b} ± 0.17	1.68^{bc} ± 0.39	10.07^{c} ± 6.99	5.04^{c} ± 0.98	14.58^{b} ± 0.4	3.12^{c} ± 0.45
Average	0.97	23.40	0.22	1.67	6.61	4.94	11.91	3.43

Values are mean ± standard deviation of three (n = 3) measurements. The superscript letters (a-c) in a row for nutritional elements present significantly different values in five varieties of betel leaves (p < 0.05) by Duncan's multiple range tests

1.3.4. Trace mineral elements in FBL

In this analysis, the trace elements were considered to have concentrations on average less than 500 µg/kg. Trace elements were reported in variable concentrations in five varieties of betel leaf. Seven trace elements, including Be, Co, Ga, Li, V, Se, and Cs were considered nontoxic trace elements from literature (Bhattacharya et al., 2016; Khan et al., 2014[c]). Their concentration ranges were reported in the decreasing order of betel leaves: Ga (380.86 – 884.75 µg/kg) > Li (27.77 – 439.72 µg/kg) > V (33.98 – 61.19 µg/kg) > Cs (10.61 – 47.33 µg/kg) > Se (14.06 – 64.72 µg/kg) > Co (15.62 – 35.47 µg/kg) > U (2.69 – 35.87 µg/kg) > and Be (0.79 -4.29

µg/kg) (Table 1.9). The analyzed trace elements had variable quantities largely dependent upon betel leaves varieties. They may not cause any adverse health impacts to the consumers.

Table 1.9. Determination of the trace element in FBL from Bangladesh

Unit: µg/kg

Betel varieties	Li	Be	V	Co	Ga	Se	Cs	U
Bangla	86.75^{a} ± 18.73	2.17^{ab} ± 1.40	61.19^{b} ± 23.31	18.68^{a} ± 1.79	461.17^{ab} ± 101.13	19.76^{a} ± 2.44	47.33^{c} ± 4.88	35.87^{b} ± 28.41
Sanchi	101.1^{a} ± 6.2	0.79^{a} ± 0.14	33.98^{a} ± 0.61	15.62^{a} ± 0.46	380.86^{a} ± 14.05	14.06^{ab} ± 3.39	45.39^{c} ± 2.02	3.35^{a} ± 0.28
Misti	114.84^{a} ± 13.92	4.29^{b} ± 0.27	48.26^{bc} ± 2.28	27.80^{b} ± 1.41	516.37^{ab} ± 19.00	36.30^{c} ± 4.03	26.00^{b} ± 0.94	3.41^{a} ± 0.18
Khasia	27.77^{a} ± 3.55	3.69^{b} ± 0.20	45.00^{bc} ± 2.08	20.68^{ab} ± 0.24	884.75^{c} ± 51.85	64.72^{d} ± 4.02	10.61^{a} ± 0.28	2.69^{a} ± 0.21
BARI Paan 3	439.72^{b} ± 316.62	3.59^{b} ± 2.01	47.21^{bc} ± 13.9	35.47^{c} ± 8.72	696.88^{bc} ± 298.93	21.85^{b} ± 3.51	32.12^{b} ± 7.98	3.97^{a} ± 1.56
Average	154.04	2.90	47.13	23.65	588.01	31.34	32.29	9.86

Values are mean ± standard deviation of three (n = 3) measurements. The superscript letters (a-c) in a row for nutritional elements present significantly different values in FBL ($p < 0.05$) by Duncan's multiple range tests

1.3.5. Toxic mineral elements in FBL

Among the toxic element, including Pb, As, Cd, and TI are considered for food due to their toxicity to human beings and other mammals (Tamele and Loureiro, 2020). Toxic element concentration monitoring after regular intervals is very important, especially in food samples. Betel leaves are one of the most important food samples because more than 600 million people consume these leaves worldwide (Pradhan et al., 2014). In this study, toxic trace elements concentration were reported for the analyzed betel leaves decreasing order were: Pb (129.36 – 965.91 µg/kg) > As (29.31 – 40.74 µg/kg) > Cd (4.94 – 15.15 µg/kg) > TI (0.52 – 1.42 µg/kg) (Table 1.10). According to FAO/WHO (1984), the permissible limit of As, Pb, and Cd in edible plants is 100 µg/kg, 300 µg/kg, and 200 µg/kg, individually. As and Cd concentration is below

the permissible limit in betel leaves, Pb concertation shows the above permissible limit in Bangla, Misti, and BARI paan 3 betel leaf varieties.

Table 1.10. Determination of the toxic element in FBL from Bangladesh

Unit: µg/kg

Betel varieties	As	Cd	TI	Pb
Bangla	$30.41^{ab} \pm 7.59$	$7.02^{a} \pm 0.73$	$1.42^{a} \pm 0.71$	$965.91^{d} \pm 142.69$
Sanchi	$29.31^{a} \pm 2.95$	$10.02^{b} \pm 0.30$	$0.52^{a} \pm 0.02$	$129.36^{a} \pm 18.44$
Misti	$37.16^{ab} \pm 4.90$	$15.15^{c} \pm 0.85$	$0.75^{a} \pm 0.10$	$763.96^{c} \pm 63.08$
Khasia	$36.37^{ab} \pm 2.70$	$4.94^{a} \pm 0.10$	$0.71^{a} \pm 0.10$	$178.62^{a} \pm 9.34$
BARI Paan 3	$40.74^{b} \pm 7.76$	$10.95^{b} \pm 2.66$	$1.24^{a} \pm 1.06$	$568.36^{b} \pm 124.98$
Average	34.80	9.61	0.93	521.24

Values are mean ± standard deviation of three (n = 3) measurements. The superscript letters (a - e) in a row for nutritional elements present significantly different values in FBL ($p < 0.05$) by Duncan's multiple range tests

1.3.6. Multivariate statistical analysis based on mineral element results in FBL

To determine the similarity and to classify the betel leaf varieties based on the identified mineral elements, principal component analysis (PCA) and hierarchical cluster analysis (HCA) were applied. PCA was used to decrease the dimension of data variance, which was calculated based on the correlation of mineral elements contents. Also, as a method for grouping, cluster analysis can be utilized where there are no clear or known classification criteria (Rahman and Rahman, 2020; Granato et al., 2018). In PCA analysis, there are four eigenvalues higher than 1, which means that there could be four principal components for data analysis after the reduction dimension. However, the two principal components, i.e., PC1 (66.8%) and PC2 (32.7%) were used to simplify the statistical analysis and obtain a PCs planer score plot. According to Figure 1.3, PC1 and PC2 can explain 99.5% of the data variance, which means some information about the nutritional elements has been lost during the statistical re-modeling. Among the nutritional elements, potassium was the highest correlation based on loading of PC1 at 0.988. Oppositely, calcium and magnesium were the maximum correlation based on loading of PC2 at 0.903 and

0.391, respectively. The analyzed betel leaves formed into three clusters in the score plot of principal component analysis: Sanchi formed cluster I; Bangla and Misti formed cluster II; BARI Paan 3 and Khasia formed cluster III (Figure 1.3). The hierarchical cluster analysis was also applied based on the amount of the 25 mineral elements identified. The Ward's method was used between group linkage and the similarity between clusters was reported as a proximate analysis. The FBL also formed three clusters in the dendrogram (Figure 1.4): Sanchi betel leaves formed cluster I; Bangla and Misti betel leaf gave cluster II; and BARI Paan 3 and Khasia betel leaf varieties formed cluster III.

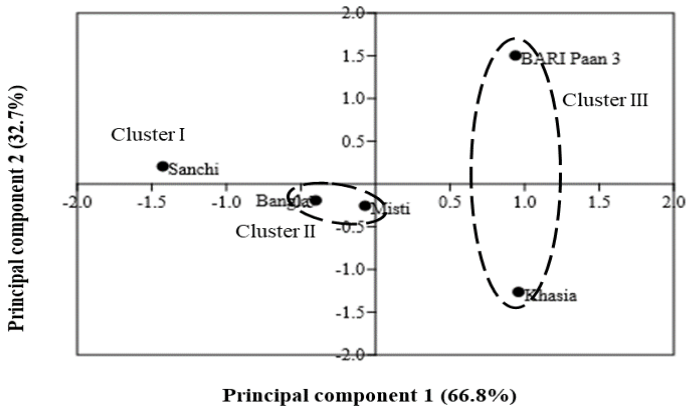

Figure 1.3. Score plot of two principal components analysis based on elemental concentration in FBL from Bangladesh.

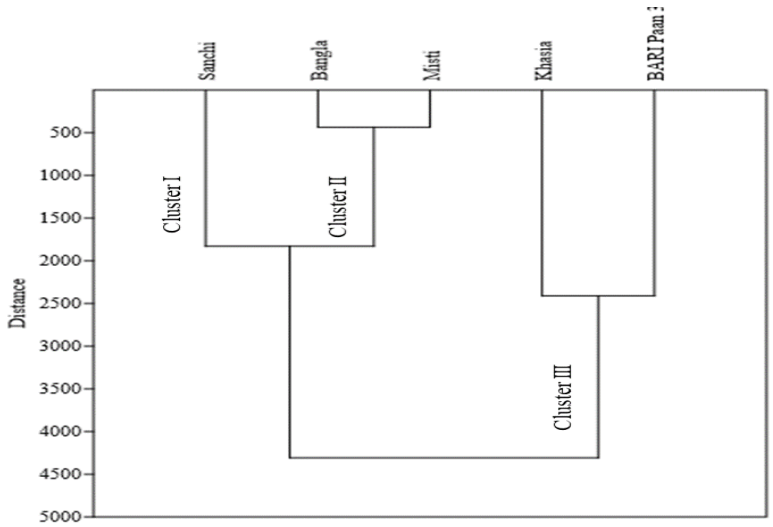

Figure 1.4. Dendrogram from all elemental concentrations in FBL from Bangladesh**.**

1.4. Conclusion

The analytical method's validation parameters were confirmed by the application of ICP-OES and ICP-MS, respectively, for the analysis of major, minor, trace, and toxic elements in FBL. The target species were found to be contributing good nutritional values of potassium, calcium, zinc, manganese, and copper to the overall intake of consumers. According to World Health Organization (WHO)/Food and Agriculture Organization (FAO), the concentration ranges of all analyzed elements were detected within the safe limit except Pb and Mn. From the multivariate chemometric analysis, the PCA and HCA were applied on all analyzed elements, followed by successful separation and classification into three clusters; for example, Sanchi formed cluster I; Bangla and Misti formed cluster II; BARI Paan 3 and Khasia formed cluster III.

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CHAPTER II

Analysis of volatile organic compounds in five varieties of *Piper betle* L. leaves (FBL) from Bangladesh

2.1. Introduction

The aroma and bioactivity of betel leaves mainly depend on the volatile organic compounds present in their essential oil (Sucipto et al., 2017). Essential oils are naturally occurring hydrophobic liquids, holding complex mixtures of volatile organic compounds (VOC's). The maximum end product of these oils secondary metabolism is terpenoids. Terpenoids are classified as monoterpenes (hydrocarbon and oxygenated monoterpenes), sesquiterpenes (hydrocarbon and oxygenated sesquiterpenes), diterpenes, and other derivatives of aromatic compounds also found in essential oil. Additionally, they contain phenolic compounds, which are derived via the shikimate pathway. They possess antibacterial, anti-fungal, gastroprotective, anti-allergic, anti-inflammatory, therapeutic, anticancer, and anti-tumor properties (Shah et al., 2016). The therapeutic oil quality depends on their different aroma chemical concentrations. The essential oil of molecular structure is very small, permitting absorption into our body's different parts. Betel leaves essential oil is slightly greasy, viscous, and slippery liquid at room temperature and it has a strong, pungent aromatic flavor with various phytochemicals such as eugenol, chavicol, methyl eugenol, chavibetol, hydroxychavicol, hydroxycatechol, β-caryophyllene, estragole, 1,8-cineol, α-pinene, β-pinene, and others. It has many applications in the food, cosmetics, and pharmaceutical industries (Guha and Nandi, 2019).

2.1.1. Importance of volatile organic compound analysis in *Piper betle* L. leaves

The demand for aromatic and medicinal plants increases daily due to consumer interest in which plants have pharmaceutical, medicinal, and other anthropogenic applications (Dhifi et

al., 2016). Consumer interest depends on important bioactive metabolites containing in medicinal plants. Since ancient times, volatile organic compounds from plants, especially from leaves, fruits, and flowers. Over ten thousand compounds have been described and associated with order characteristics in flavor chemistry, usually classified into different chemical groups, for example, carbonyl compounds, terpenes, esters, alcohol, ketone, and etc. Human-related studies of volatile compounds have ranged from basic olfactory studies to investigations of therapeutic properties, physiological and psychological effects. Betel leaves produce a great variety of secondary metabolites. The composition of volatile organic compounds affects aromatic herbs and essential oil's bioactivity. The types of volatile compounds and amounts depend on the growing condition, plant varieties, environmental factors, and etc. However, there were no reports available on a comparative study of volatile organic compounds in five varieties of betel leaf (Bangla, Sanchi, Misti, Khasia, and BARI Paan 3) from Bangladesh.

2.1.2. Analysis of volatile organic compounds

The analytical procedure of volatile organic compounds from plant matrices includes two steps. One step is extraction (hydro-distillation, steam distillation, simultaneous distillation extraction, supercritical fluid extraction, and etc) and the other step is an analysis of this extraction used by gas chromatography (GC), or gas chromatography-mass spectrometry (GC-MS). In this study, the using method is simultaneous distillation extraction (SDE) and analyzed by GC-MS (Swift et al., 2012).

2.1.2.1. Simultaneous distillation extraction

Simultaneous distillation extraction (SDE) is known as Lickens-Nickerson method. This method is famous for isolation techniques in flavor and fragrance laboratories, which combine the volatility and extractability into a single step (Swift, 2012). The original apparatus has been modified by Schultz et al. (1977) to improve the recoveries and to use small quantities of

materials. SDE is one-step isolation and concentration of constituents of flavor that works by dividing the compounds of non-polar flavor into a water-immiscible solvent. The recycling of organic solvent and water allows the SDE system. It is a time-saving method of preparing the sample and using a less organic solvent, which saves a lot of analytical costs (Chaintreau, 2001). The distillate-return arm's different heights allow both less than water solvents and denser than water solvents to be used by the apparatus. The extraction takes place by putting the sample first in a round bottom flask and then mixing it with water and bringing it to boil. The volatile organic compounds are stem distilled through the upper portion of the suitable arm, while the organic solvent vapors are also distilled through the other arm at the same time. The middle chamber enables both stem-distilled vapor and organic solvent vapors to be combined, which condense into the separator together on the cold finger, where the emulsion of polar and polar phases separates before recycling back to their respective flasks (Figure 2.1).

The rate of solvent flow of their vapors through the system's necks and interacting depends on their temperature and is a significant factor in the SDE (Careri et al., 1999). The rate at which each phase is volatilized and condensed may alter the ratio of cold finger condensing solvents and may eventually alter the phase emulsion's separation properties in the SDE separation portion. The rise in steam and solvent vapor flows indicated a higher rate of compound recovery (Pollien et al., 1997). SDE has been found to have varying percentages of recovery depending on a variety of factors, but mainly the extraction time, pH, solvent types, type of condenser used, atmospheric pressure, volatile sample concentration, salt concentration, and temperature of the cold finger (Chaintreau et al., 2001; Careri et al., 1999). The types of compounds analyzed after extraction and concentration using SDE are also of concern as thermolabile compounds can decompose. The higher temperature at which SDE occurs promotes a greater tendency to volatile oxidation (Gu et al., 2009).

Despite the disadvantages of SDE, high recovery yields with low variability from a variety of matrices and the ability to model the loss of solvent during an experiment by adding an internal standard to the sample solidify its use in flavor research (Godefroot et al., 1981). In the separation, qualitative analysis, and quantitative analysis of volatiles from several types of materials, the addition of an internal standard has allowed in SDE. Recovered isolates containing nearly all the volatile compounds as the starting materials without interfering lipid-containing compounds obtained in solvent extraction are another reason that SDE is a suitable method of volatile extraction (Pollien et al., 1997).

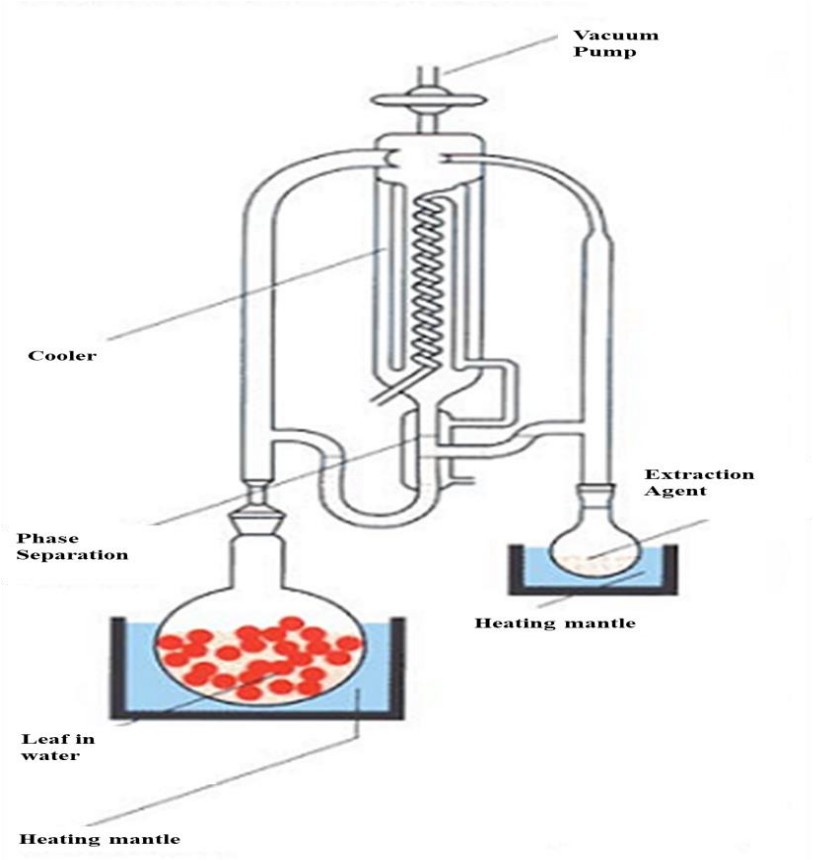

Figure 2.1. Apparatus of simultaneous steam distillation (SDE) according to Lickens-Nickerson.

2.1.2.2. Gas chromatography-mass spectrometry (GC-MS)

Gas-chromatography (GC) is an important analytical technique used to analyze volatile organic compounds in foods and other agricultural products. A mixture of volatile organic compounds is separated and analyzed in a food sample without GC decomposition (Azarnia et al., 2012). In gas chromatography, the carrier gas or mobile phase is an inert gas. For example, helium and the stationary phases are very thin polymer or liquid layers on inert solid support inside a column. The volatile organic compounds interact with the column walls, and these are eluted based on the temperature of the column at definite retention times (Grob, 2004). The eluted organic compounds are identified with detectors. Mass spectrometry and flame ionization are the most suitable detector to detect volatile organic compounds (Vas and Vekey, 2004).

Mass spectrometry (MS) is an analytical technique that produces a single spectrum of the masses of the molecules or atoms comparing a sample of material. Samples (solid, liquid, or gas) are ionized by bombarding electrons. MS is worked by ionizing chemical compounds to generated molecular fragments or charged molecules and measuring their mass-to-charge ratios (Luedemann et al., 2008). The ions are detected by a mechanism capable of detecting charged particles, for example, an electron multiplier. As a result, it is displaced as spectra of the relative abundance in the sample can be identified by comparing known masses to the identified masses or follow a characteristics pattern (Sparkman and David, 2006).

2.1.3. Justification of this study

A wide variety of volatile organic compounds were found in the betel leaves, which vary depending on the type of landraces, growing location, environment, and soil (Suryasnata et al., 2016). Different extraction methods (hydro distillation, steam distillation, supercritical fluid extraction, and solvent extraction) were used to extract volatile organic compounds from betel leaves, which work for any other essential oil-bearing crops. The simultaneous distillation extraction (SDE) technique is usually considered superior to classical ones in the flavor and

fragrance area, like solvent extraction or distillation. SDE technique has been developed to isolate volatile organic compounds, introduced in 1964 by Likens and Nickerson (Nickerson and Likens, 1996). SDE technique has been successfully applied in the extraction of aroma compounds, essential oils, and other volatile organic compounds from various matrices (Chaintreau, 2001). As far as the authors know, the SDE technique to isolate betel leaves volatile compounds has not been reported before. Similarly, no report has been found on the volatile compound analysis of betel leaves that grow in Bangladesh with SDE and/or other methods. Therefore this study was designed to investigate volatile compounds in five common varieties of betel leaves that grow in Bangladesh. The volatile organic compound extraction was carried out using SDE with *n*-pentane:diethyl ether (1:1) solvent and analyzed by GC-MS. Subsequently, the PCA and HCA were used to classify the analyzed samples using the volatile compounds identified as fingerprints. Comparing the composition of volatile compounds among the Bangladeshi betel leaf varieties tried to understand each analyzed subject species medicinal properties.

2.2. Materials and Methods

2.2.1. Sample collection

See details in chapter 1, section 1.2.1.

2.2.2. Reagent and chemicals

All analytical grade reagents and chemicals were used in this analysis. The organic solvents such as *n*-pentane, diethyl ether, and anhydrous Na_2SO_4 were purchased from Fisher Scientific (Waltham, Massachusetts, USA) and sigma company (St Louis, Mo, USA).

2.2.3. Analytical apparatus of volatile organic compounds

The organic volatile compound extraction of *Piper betle* L. related apparatus details are shown in Table 2.1.

Table 2.1. Volatile organic compound extraction and analysis related apparatus list

S. N.	Apparatus name	Description
i.	Round bottom flask	2.5 L capacity
ii.	Furnace	F 6000, Barnstead Thermolyne Co., IA, USA)
iii.	pH meter	pH meter (HM-30P, DKK-TOA Corp., Tokoyo, Japan)
iv.	Distilling apparatus	Wire spiral packed double distilling apparatus (Normschliff Geratebau, Germany)
v.	Extraction apparatus	Simultaneous steam distillation and extraction (SDE), Likens & Nickerson type simultaneous steam distillation & extraction apparatus (Normschliff, werthein, Germany)
vi.	Concentration column	Vigreux column (250 mL, Normschliff, werthein, Germany)
vii.	Capillary column	ZB-5MS (60 m length, 0.25 mm diameter, 0.25 µm film thickness, J & W, USA)

2.2.4. Extraction of volatile compounds FBL

Each 10 g powder sample was mixed with 1 L of distilled water and then the p^H was adjusted to 7.0 by adding dilute NaOH or HCl solution. Then 10 mL (110 ppm in *n*-pentane) *n*-butyl benzene was added as an internal standard for the quantitative analysis of volatile compounds. Volatile organic compounds were extracted from betel leaves for 3 hours with 100 mL redistilled *n*-pentane:diethyl ether (1:1, v/v) mixture using a simultaneous distillation extraction (SDE, Likens & Nickerson types) apparatus as modified by Schultz et al., 1977 and

the experiment was continued under normal atmospheric pressure. The extract was dehydrated for 12 hours with anhydrous Na_2SO_4 and concentrated to a final volume of approximately 1.5 mL using a Vigreux column. After that, it was further concentrated (0.5 mL) under N_2 gas mild flush. Finally, the concentrated extract was injected into the GC-MS system.

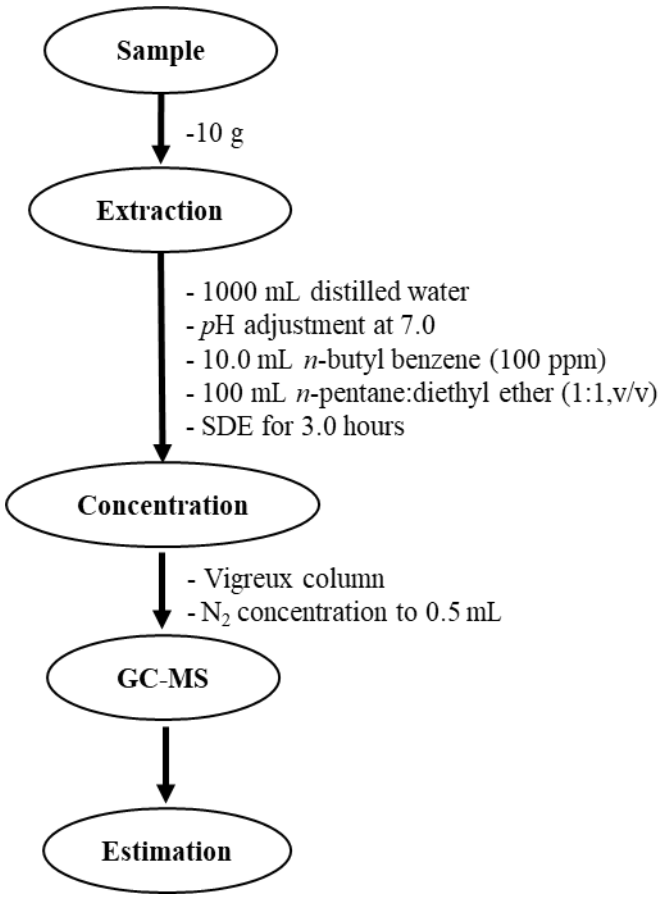

Figure 2.2. Extraction scheme for volatile organic compounds in FBL from Bangladesh.

2.2.5. Establishment of retention index

Kovats suggested that retention index (RI) or Kovats index (KI) is a suitable identification rule. The same compound indicated retention index to retention time for a standard alkane. RI was used as a parameter for checking a solute from chromatogram by comparing the retention time (RT) of both alkane and solute that appeared above and below the solute (Davies, 1990).

$$RI_i = 100\,Z + 100\,\{\mathrm{Log}\,V_{R(i)} - V_{R(Z)} / V_{R(Z+1)} - V_{R(Z)}\} \quad (2.1)$$

Here,

RI_i = Retention index of compound i

$V_{R(i)}$, $V_{R(Z)}$, $V_{R(Z+1)}$ = Retention time of standard alkanes (alkanes eluate before and after the factor, Z = Factor Z contains the number of carbon eluate Z+1, Z+2…….etc

RT of an alkane is the values as several of the carbon number that the compound has to be unrelated with solid column phase, the temperature of separation, and other chromatography requirements. As a result, *n*-alkane was indicated as a standard index for CH_4 (RI = 100), C_2H_6 (RI = 200),…….C_nH_{2n+2} (RI = 100_n), and even anything in the analysis column. A scaled RT of the standard sample of a known hydrocarbon mixture of *n*-alkane ($C_8 \sim C_{22}$) was used as a standard. 1 µL mixture was analyzed to determine the RT of the standard (*n*-alkane) by GC-MS under the same conditions was applied for betel leaves sample extract analysis. RI of each peak was established by a basic program that substituent the RT of each peak of *n*-alkane confirmed by GC chromatogram.

2.2.6. Analysis of volatile organic compounds by GC-MS

Agilent 7890 gas chromatograph (GC) coupled with Agilent 7000 mass spectrometer (MS) (Agilent Technologies, Santa Clara, CA, USA) and the EI (electron impact) mode was used for the quantitative analysis of the volatile compound. The ionization voltage, the temperature of the injector, and ion source temperature were 70 eV, 250 ºC, and 220 ºC, respectively. The mass

spectra were scanned from 50 to 400 m/z. A ZB-5MS capillary column (60 m ×0.25 mm i.d., 0.25 µm film thickness, Phenomenex, USA) was used for the separation of volatile compounds. The oven temperature was programmed as follows, 40 ºC (isothermal for 3 min) which was increased to 220 ºC at 2 ºC min^{-1} and then to 300 ºC at 10 ºC min^{-1} (isothermal for 10 min). Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1} with an injector volume of 1 µL using a 1:20 split ratio. The betel leaves volatile organic compounds were identified by our own mass spectral database and the spectral libraries including FFNSC (2012), NIST 12 and WILEY 7 were provided with the GC-MS instrument and mass spectral data books (Davies, 1990). Additionally, comparisons of retention indices to reference data were considered (Adams, 2007). The linear retention index was used as a parameter by comparing the retention time of solutes in chromatography, where standard *n*-alkanes (C_8 - C_{22}) were used as an external reference. The quantitative analysis of volatile compounds was carried out with the help of peak area percent of an internal standard by using the following formula:

$$\text{Volatile amount (mg/kg)} = \frac{C \times 1000}{A \times B} \quad \text{.....(2.2)}$$

Here, A = Peak area of each sample in the internal standard

B = Amount of sample (g)

C = Peak area of each component in the sample

2.2.7. Statistical analysis

The volatile compounds were determined in triplicate, and the data evaluated using Statistical Package for Social Sciences (SPSS), Software Version 20 (IBM, New York, USA). The results were reported as mean ± standard deviation (mg/kg) on a dry weight basis. Principal component analysis (PCA) and Hierarchical cluster analysis (HCA) were worked by PAST (version 3.25) software.

2.3. Results and Discussion

2.3.1. Establishment of retention index of *n*-alkane

Retention Index (RI) value was determined by *n*-alkane mixture (C_8 ~ C_{22}), considering as standard. 1 µL *n*-alkane mixture was analyzed to determine the retention time (RT) by GC-MS. The analytical condition of GC-MS was the same as the sample analysis condition. The retention index of each peak was established with the help of a basic program as mentioned in section 2.2.5. The GC-MS chromatogram confirmed that the RT of each peak of *n*-alkane (Table 2.2. and Figure 2.3)

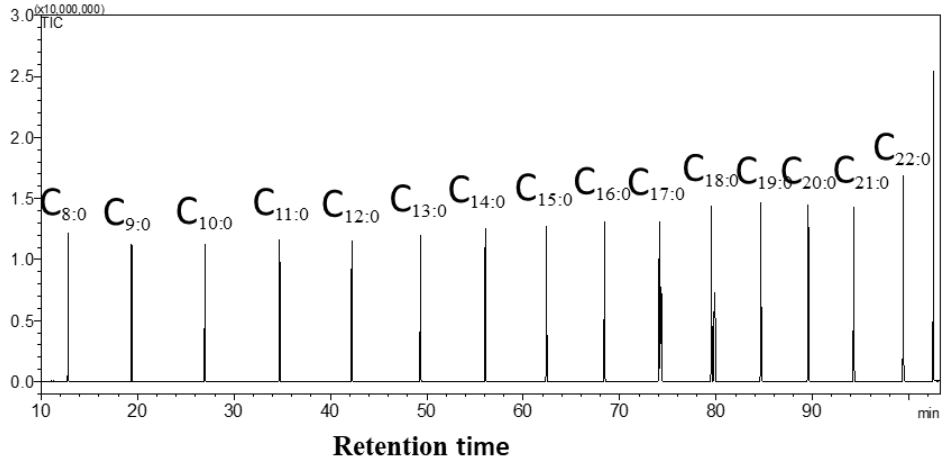

Figure 2.3. GC-MS chromatograms of *n*-alkane standard mixture (C_8 ~ C_{22}).

Table 2.2. Retention time and retention index of *n*-alkane for GC-MS

Alkanes	RT[1]	RI[2]	Alkanes	RT	RI
$C_{8:0}$	12.812	800	$C_{16:0}$	68.425	1600
$C_{9:0}$	19.398	900	$C_{17:0}$	74.117	1700
$C_{10:0}$	26.992	1000	$C_{18:0}$	79.522	1800
$C_{11:0}$	34.733	1100	$C_{19:0}$	84.660	1900
$C_{12:0}$	42.227	1200	$C_{20:0}$	89.562	2000
$C_{13:0}$	49.345	1300	$C_{21:0}$	94.283	2100
$C_{14:0}$	56.068	1400	$C_{22:0}$	99.408	2200
$C_{15:0}$	62.420	1500			

[1]RT = Retention time; [2]RI = Retention index

2.3.2. Volatile organic compounds in FBL

The average volatile organic compounds concentration was shown as mean ± standard deviation of three replicates of five varieties *Piper betle* L. (Bangla, Sanchi, Misti, Khasia, and BARI Paan 3) from Bangladesh. All the compounds have been numbered according to their elution order. These were discussed separately for each betel leaves ingredient under the following headings.

2.3.2.1. Volatile organic compounds in *Piper betle* L. var. Bangla

As shown in Table 2.3, our study confirms that the amount of volatile compounds was 4346.91 mg/kg in Bangla, while 72 compounds belongs to different chemical functional group were identified, including 24 hydrocarbons (13.47 %, 585.44 mg/kg), 15 alcohols (80.44 %, 3496.55 mg/kg), 11 aldehydes (0.87 %, 37.82 mg/kg), 9 esters (1.93 %, 84.02 mg/kg), 7 ethers (3.06 %, 133.01 mg/kg) and 6 ketones (0.23 %, 10.00 mg/kg) (Table 2.4). Terpene group was the dominant chemical class with the highest proportion (95.52 %, 4152.07 mg/kg), while the oxygenated monoterpene, sesquiterpene hydrocarbon, oxygenated sesquiterpene, monoterpene hydrocarbon and oxygenated diterpene were (79.51 %, 3456.10 mg/kg), (12.96 %, 563.48 mg/kg), (2.53 %, 109.84 mg/kg), (0.51 %, 21.96 mg/kg) and (0.02 %, 0.69 mg/kg), respectively (Table 2.5). The major volatile compounds were eugenol (78.52 %, 3412.97 mg/kg) after that γ-muurolene (6.45 %, 280.36 mg/kg), β-caryophyllene (2.64 %, 114.69 mg/kg), eugenol acetate (1.50 %, 65.38 mg/kg), α-hemulene (1.35 %, 58.77 mg/kg) and caryophyllene oxide (1.34 %, 58.26 mg/kg). Two compounds, oxophorone and 9-epi-β-caryophyllene were found only in Bangla betel leaf (Table 2.18).

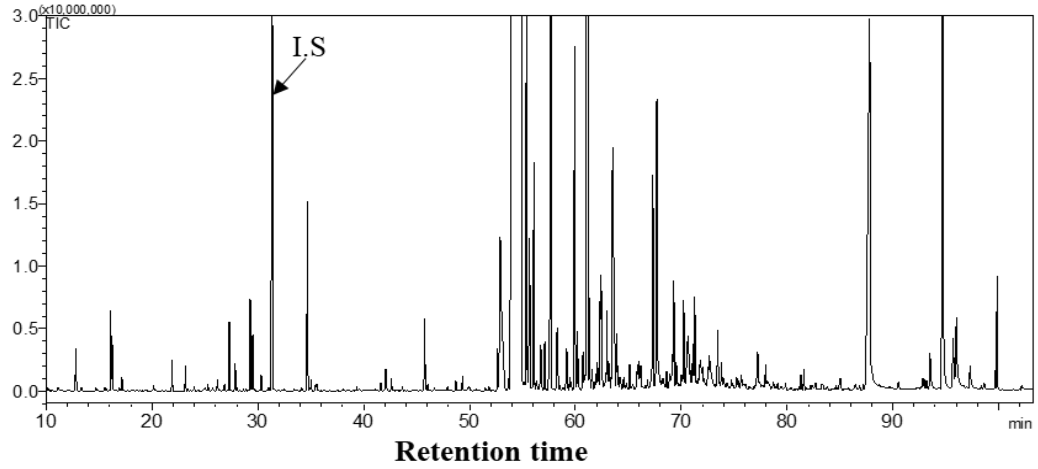

Figure 2.4. GC-MS chromatogram of volatile organic compounds of *Piper betle* L. var Bangla from Bangladesh, I.S. = Internal standard (*n*-butyl benzene).

Table 2.3. Volatile organic compounds identified in *Piper betle* L. var. Bangla from angladesh

Peak no	RT	RI	Compound name	MF	MW	Area (%)	Amount (mg/kg)
1	8.233	730	**2-Ethylfuran**[f]	C_6H_8O	96	0.22 ± 0.00	9.41 ± 0.30
2	11.200	776	**2-Penten-1-ol**[a]	$C_5H_{10}O$	86	0.01 ± 0.00	0.27 ± 0.01
3	12.725	799	**3-Hexenal**[b]	$C_6H_{10}O$	98	0.05 ± 0.00	2.05 ± 0.05
4	12.825	800	**Hexanal**[b]	$C_6H_{12}O$	100	0.12 ± 0.00	5.12 ± 0.06
5	14.683	828	Furfuraldehyde[b]	$C_5H_4O_2$	96	0.01 ± 0.00	0.47 ± 0.01
6	16.100	850	**2-Hexenal**[b]	$C_6H_{10}O$	98	0.24 ± 0.00	10.29 ± 0.17
7	16.225	852	3-Hexen-1-ol[a]	$C_6H_{12}O$	100	0.15 ± 0.00	6.32 ± 0.10
8	16.933	863	**2-Hexen-1-ol**[a]	$C_6H_{12}O$	100	0.01 ± 0.00	0.44 ± 0.02
9	17.167	866	**1-Hexanol**[a]	$C_6H_{14}O$	102	0.04 ± 0.00	1.84 ± 0.03
10	21.892	933	*α*-Pinene[d]	$C_{10}H_{16}$	136	0.09 ± 0.00	4.09 ± 0.05
11	23.158	950	Camphene[d]	$C_{10}H_{16}$	136	0.08 ± 0.00	3.56 ± 0.05
12	23.983	960	**Benzaldehyde**[b]	C_7H_6O	106	0.01 ± 0.00	0.49 ± 0.01
13	25.283	977	*β*-Pinene[d]	$C_{10}H_{16}$	136	0.02 ± 0.00	0.79 ± 0.01
14	25.733	983	Methyl heptenon[e]	$C_8H_{14}O$	126	0.01 ± 0.00	0.61 ± 0.01
15	26.192	989	**2-pentylfuran**[f]	$C_9H_{14}O$	138	0.04 ± 0.00	1.79 ± 0.02
16	27.300	1004	*(Z)*-3-Hexenyl-1-acetate[c]	$C_8H_{14}O_2$	142	0.22 ± 0.00	9.50 ± 0.15
17	27.860	1011	*n*-Hexyl acetate[c]	$C_6H_{16}O_2$	100	0.09 ± 0.00	3.88 ± 0.07
18	28.050	1014	*(E)*-2-Hexenyl acetate[c]	$C_8H_{14}O_2$	142	0.01 ± 0.00	0.38 ± 0.01
19	28.892	1025	*P*-cymene[d]	$C_{10}H_{14}$	134	0.01 ± 0.00	0.26 ± 0.01
20	29.275	1029	Limonene[d]	$C_{10}H_{16}$	136	0.31 ± 0.00	13.27 ± 0.16

Peak no	RT	RI	Compound name	MF	MW	Area (%)	Amount (mg/kg)
21	29.517	1033	Eucalyptol[f]	$C_{10}H_{18}O$	154	0.18 ± 0.00	7.91 ± 0.10
22	30.317	1043	**Phenyl acetaldehyde[b]**	C_8H_8O	120	0.05 ± 0.00	2.39 ± 0.02
I.S.	31.350	1056	*n*-butyl benzene	C_9H_{14}	-	-	-
23	34.675	1099	Linalool[a]	$C_{10}H_{18}O$	154	0.66 ± 0.01	28.53 ± 0.65
24	35.042	1104	**Nonanal[b]**	$C_9H_{18}O$	142	0.03 ± 0.00	1.40 ± 0.03
25	35.558	1111	**Phenylethyl alcohol[a]**	$C_8H_{10}O$	122	0.01 ± 0.00	0.38 ± 0.02
26	38.042	1144	**Oxophorone[e]**	$C_9H_{12}O_2$	152	0.01 ± 0.00	0.26 ± 0.01
27	39.350	1162	Benzyl acetate[c]	$C_9H_{10}O_2$	150	0.01 ± 0.00	0.39 ± 0.01
28	41.633	1192	Methyl salicylate[c]	$C_8H_8O_3$	152	0.03 ± 0.00	1.17 ± 0.02
29	42.075	1198	Estragole[f]	$C_{10}H_{12}O$	148	0.08 ± 0.00	3.34 ± 0.07
30	42.625	1206	Decanal[b]	$C_{10}H_{20}O$	156	0.04 ± 0.00	1.88 ± 0.06
31	43.650	1220	***β*-Cyclocitral[b]**	$C_{10}H_{16}O$	152	0.01 ± 0.00	0.51 ± 0.01
32	45.758	1250	Chavicol[a]	$C_9H_{10}O$	134	0.26 ± 0.00	11.39 ± 0.23
33	46.050	1254	**2-Phenylethyl acetate[c]**	$C_{10}H_{12}O_2$	164	0.02 ± 0.00	0.97 ± 0.02
34	47.933	1280	**Linalool oxide acetate[c]**	$C_{12}H_{20}O_3$	212	0.05 ± 0.00	2.00 ± 0.05
35	49.958	1309	**4-vinyl Guaiacol[a]**	$C_9H_{10}O_2$	150	0.01 ± 0.00	0.53 ± 0.01
36	54.162	1372	Eugenol[a]	$C_{10}H_{12}O_2$	164	78.52 ± 0.27	3412.97 ± 1.31
37	55.675	1394	***β*-Elemene[d]**	$C_{15}H_{24}$	204	0.6 ± 0.01	26.24 ± 0.56
38	56.083	1400	Methyleugenol[d]	$C_{11}H_{14}O_2$	178	0.81 ± 0.01	35.18 ± 0.69
39	56.733	1410	***n*-Dodecanal[b]**	$C_{12}H_{24}O$	184	0.19 ± 0.00	8.32 ± 0.20
40	57.108	1416	***(Z)*-α- Bergamotene[d]**	$C_{15}H_{24}$	204	0.17 ± 0.00	7.26 ± 0.18
41	57.700	1426	*β*-Caryophyllene[d]	$C_{15}H_{24}$	204	2.64 ± 0.04	114.69 ± 2.24
42	58.533	1439	*α*-Guaiene[d]	$C_{15}H_{24}$	204	0.01 ± 0.00	0.56 ± 0.01
43	58.833	1445	**9-epi-β-Caryophyllene[d]**	$C_{15}H_{24}$	204	0.01 ± 0.00	0.51 ± 0.04
44	59.175	1449	**Isogermacrene D[d]**	$C_{15}H_{24}$	204	0.18 ± 0.00	7.94 ± 0.18
45	59.933	1461	*α*-Humulene[d]	$C_{15}H_{24}$	204	1.35 ± 0.02	58.77 ± 1.25
46	60.858	1475	**Cadina-1(6),4-diene[d]**	$C_{15}H_{24}$	204	0.02 ± 0.00	0.69 ± 0.03
47	61.142	1480	*γ*-Muurolene[d]	$C_{15}H_{24}$	204	6.45 ± 0.10	280.36 ± 5.40
48	61.325	1483	*α*-Curcumene[d]	$C_{15}H_{22}$	202	0.31 ± 0.00	13.31 ± 0.36
49	61.583	1487	***β*-(Z)-Bergamotene[d]**	$C_{15}H_{24}$	204	0.09 ± 0.00	3.90 ± 0.10
50	61.892	1492	**Eremophilene[d]**	$C_{15}H_{24}$	204	0.02 ± 0.00	0.97 ± 0.04
51	62.025	1494	*β*-Selinene[d]	$C_{15}H_{24}$	204	0.07 ± 0.00	2.86 ± 0.03
52	62.192	1496	***γ*-Amorphene[d]**	$C_{15}H_{24}$	204	0.05 ± 0.00	1.98 ± 0.06
53	62.367	1499	Cubebol[a]	$C_{15}H_{26}O$	222	0.42 ± 0.01	18.44 ± 0.61
54	62.483	1501	*α*-Muurolene[d]	$C_{15}H_{24}$	204	0.36 ± 0.01	15.83 ± 0.53
55	63.517	1518	Eugenyl acetate[c]	$C_{12}H_{14}O_3$	206	1.50 ± 0.00	65.38 ± 0.60
56	63.783	1523	*δ*-Cadinene[d]	$C_{15}H_{24}$	204	0.35 ± 0.00	15.24 ± 0.37
57	63.908	1525	***(E)*-Calamenene[d]**	$C_{15}H_{22}$	202	0.24 ± 0.00	10.42 ± 0.06
58	64.575	1536	***(E)*-Cadina-1,4-diene[d]**	$C_{15}H_{24}$	204	0.03 ± 0.00	1.50 ± 0.03

Peak no	RT	RI	Compound name	MF	MW	Area (%)	Amount (mg/kg)
59	66.183	1563	**1-nor-Bourbonanone[e]**	$C_{14}H_{22}O$	206	0.09 ± 0.00	4.05 ± 0.09
60	67.692	1588	Caryophyllene oxide[f]	$C_{15}H_{24}O$	220	1.34 ± 0.01	58.26 ± 0.97
61	68.258	1597	**Salvial-4(14)-en-1-one[e]**	$C_{15}H_{24}O$	220	0.03 ± 0.00	1.27 ± 0.06
62	69.158	1613	**Tetradecanal[b]**	$C_{14}H_{28}O$	212	0.11 ± 0.00	4.93 ± 0.08
63	69.292	1615	**Humulene epoxide II[f]**	$C_{15}H_{24}O$	220	0.39 ± 0.00	17.12 ± 0.35
64	71.275	1650	*α*-Muurolol[a]	$C_{15}H_{26}O$	222	0.02 ± 0.00	0.69 ± 0.04
65	71.800	1659	**Cadin-4-en-10-ol[a]**	$C_{15}H_{26}O$	222	0.12 ± 0.00	5.25 ± 0.09
66	72.525	1672	***β*-Bisabolol[a]**	$C_{15}H_{26}O$	222	0.09 ± 0.00	4.07 ± 0.07
67	72.758	1676	Cadalene[d]	$C_{15}H_{18}$	198	0.01 ± 0.00	0.44 ± 0.03
68	76.717	1748	*(E)*-Cadinol[a]	$C_{15}H_{26}O$	222	0.11 ± 0.01	4.75 ± 0.65
69	79.842	1806	**Farnesyl acetate[c]**	$C_{17}H_{28}O_2$	264	0.01 ± 0.00	0.37 ± 0.01
70	81.592	1840	**Phytone[e]**	$C_{18}H_{36}O$	268	0.07 ± 0.00	3.07 ± 0.06
71	85.083	1909	**Farnesyl acetone[e]**	$C_{18}H_{30}O$	262	0.02 ± 0.00	0.75 ± 0.03
72	86.858	1945	**Isophytol[a]**	$C_{20}H_{40}O$	296	0.02 ± 0.00	0.69 ± 0.01
			Total			**100.00**	**4346.91 ± 20.03**

Data were reported by mean ± standard deviation (n = 3); 0.00 = The value is less than 0.01; RT = Retention time in minutes; RI = Retention index; MF = Molecular formula; MW = Molecular weight; [a-k] Superscripts represent the functional group of compound (a = Alcohol; b = Aldehyde; c = Ester; d = Hydrocarbon; e = Ketone; f = Ether; g = Miscellaneous); I.S. = Internal standard; Bold mark compounds name = Firstly reported volatile compound in betel leaf; Apx. - = Not detected

Table 2.4. Relative content of functional group detected in *Piper betle* L. var. Bangla from Bangladesh

Functional group	No	Area (%)	Amount (mg/kg)
Alcohol	15	80.44	3496.55
Aldehyde	11	0.87	37.82
Ester	9	1.93	84.02
Hydrocarbon	24	13.47	585.44
Ketone	6	0.23	10.00
Ether	7	3.06	133.01
Miscellaneous	-	-	-
Total	**72**	**100.00**	**4346.84**

No = No of volatile compounds; - = Not determined

Table 2.5. Relative content of terpene group detected in *Piper betle* L. var. Bangla from Bangladesh

Terpene group	No	Area (%)	Amount (mg/kg)
Monoterpene hydrocarbons	5	0.51	21.96
Oxygenated monoterpene	7	79.51	3456.10
Sesquiterpene hydrocarbons	19	12.96	563.48
Oxygenated sesquiterpene	8	2.53	109.84
Oxygenated diterpene	1	0.02	0.69
Total	**40**	**95.52**	**4152.07**

No = No of volatile compounds

2.3.2.2. Volatile organic compounds in *Piper betle* L. var. Sanchi

Table 2.6 show that the amount of volatile compounds was 6958.51 mg/kg in Sanchi betel leaf. 70 volatile compounds were detected, including 24 hydrocarbons (9.34 %, 649.86 mg/kg), 13 alcohols (86.88%, 6045.60 mg/kg), 11 aldehydes (0.64 %, 44.34 mg/kg), 9 esters (0.96 %, 66.63 mg/kg), 7 ethers (2.03 %, 141.45 mg/kg), 5 ketones (0.12 %, 8.47 mg/kg) and one N-containing compound (0.03 %, 2.16 mg/kg) (Table 2.7). As per Table 2.8, terpene group (96.47 %, 6740.80 mg/kg) was the major chemical component, whereas oxygenated monoterpene (85.74 %, 5966.01 mg/kg), sesquiterpene hydrocarbon (9.05%, 629.57 mg/kg), oxygenated sesquiterpene (1.78%, 124.01 mg/kg), monoterpene hydrocarbon (0.29 %, 20.29 mg/kg) and oxygenated diterpene (0.01 %, 0.93 mg/kg) were detected. The main volatile compounds were eugenol (85.18 %, 5927.30 mg/kg) followed by γ-muurolene (4.49 %, 312.49 mg/kg), β-caryophyllene (1.74 %, 121.41 mg/kg). This variety has one (1-H-Indole) uncommon compound (Table 2.18).

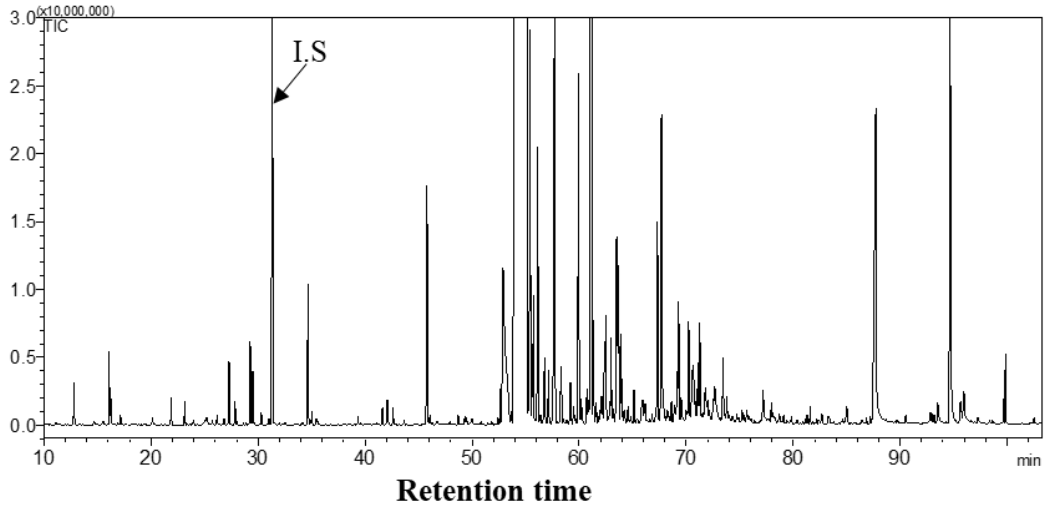

Figure 2.5. GC-MS chromatogram of volatile organic compounds in *Piper betle* L. var. Sanchi from Bangladesh I.S. = Internal standard (*n*-butyl benzene).

Table 2.6. Volatile compounds identified in *Piper betle* L. var. Sanchi from Bangladesh

Peak no	RT	RI	Compound name	MF	MW	Area (%)	Amount (mg/kg)
1	8.233	730	**2-Ethylfuran**[f]	C_6H_8O	96	0.10 ± 0.00	7.17 ± 0.10
2	11.200	776	**2-Penten-1-ol**[a]	$C_5H_{10}O$	86	0.00 ± 0.00	0.23 ± 0.00
3	12.725	799	**3-Hexenal**[b]	$C_6H_{10}O$	98	0.01 ± 0.00	0.88 ± 0.08
4	12.825	800	**Hexanal**[b]	$C_6H_{12}O$	100	0.08 ± 0.00	5.67 ± 0.51
5	14.683	828	Furfuraldehyde[b]	$C_5H_4O_2$	96	0.01 ± 0.00	0.59 ± 0.01
6	16.100	850	**2-Hexenal**[b]	$C_6H_{10}O$	98	0.14 ± 0.00	0.00 ± 0.00
7	16.225	852	3-Hexen-1-ol[a]	$C_6H_{12}O$	100	0.06 ± 0.00	4.44 ± 0.07
8	17.167	866	**1-Hexanol**[a]	$C_6H_{14}O$	102	0.02 ± 0.00	1.32 ± 0.02
9	21.892	933	*α*-Pinene[d]	$C_{10}H_{16}$	136	0.05 ± 0.00	3.78 ± 0.05
10	23.158	950	Camphene[d]	$C_{10}H_{16}$	136	0.05 ± 0.00	3.45 ± 0.04
11	23.983	960	**Benzaldehyde**[b]	C_7H_6O	106	0.01 ± 0.00	0.43 ± 0.00
12	25.283	977	*β*-Pinene[d]	$C_{10}H_{16}$	136	0.01 ± 0.00	0.48 ± 0.01
13	25.733	983	Methyl heptenon[e]	$C_8H_{14}O$	126	0.01 ± 0.00	0.69 ± 0.01
14	26.192	989	**2-pentylfuran**[f]	$C_9H_{14}O$	138	0.02 ± 0.00	1.65 ± 0.02
15	27.300	1004	*(Z)*-3-Hexenyl-1-acetate[c]	$C_8H_{14}O_2$	142	0.13 ± 0.00	9.25 ± 0.14
16	27.860	1011	*n*-Hexyl acetate[c]	$C_6H_{16}O_2$	100	0.05 ± 0.00	3.42 ± 0.04
17	28.050	1014	*(E)*-2-Hexenyl acetate[c]	$C_8H_{14}O_2$	142	0.01 ± 0.00	0.38 ± 0.01
18	29.275	1029	Limonene[d]	$C_{10}H_{16}$	136	0.18 ± 0.00	12.58 ± 0.15
19	29.517	1033	Eucalyptol[f]	$C_{10}H_{18}O$	154	0.11 ± 0.00	8.00 ± 0.11
20	30.317	1043	**Phenyl acetaldehyde**[b]	C_8H_8O	120	0.03 ± 0.00	1.82 ± 0.06

Peak no	RT	RI	Compound name	MF	MW	Area (%)	Amount (mg/kg)
I.S.	31.350	1056	*n*-Butyl benzen[e]	C_9H_{14}	134	-	-
21	34.675	1099	Linalool[a]	$C_{10}H_{18}O$	154	0.31 ± 0.00	21.9 ± 0.40
22	35.042	1104	**Nonanal[b]**	$C_9H_{18}O$	142	0.03 ± 0.00	1.86 ± 0.04
23	35.558	1111	**Phenylethyl alcohol[a]**	$C_8H_{10}O$	122	0.00 ± 0.00	0.24 ± 0.02
24	39.350	1162	Benzyl acetate[c]	$C_9H_{10}O_2$	150	0.02 ± 0.00	1.05 ± 0.01
25	41.633	1192	Methyl salicylate[c]	$C_8H_8O_3$	152	0.04 ± 0.00	2.66 ± 0.01
26	42.075	1198	Estragole[f]	$C_{10}H_{12}O$	148	0.06 ± 0.00	4.08 ± 0.08
27	42.625	1206	Decanal[b]	$C_{10}H_{20}O$	156	0.04 ± 0.00	2.70 ± 0.06
28	43.650	1220	***β*-Cyclocitral[b]**	$C_{10}H_{16}O$	152	0.01 ± 0.00	0.65 ± 0.01
29	45.758	1250	Chavicol[a]	$C_9H_{10}O$	134	0.61 ± 0.01	42.18 ± 0.79
30	46.050	1254	**2-Phenylethyl acetate[c]**	$C_{10}H_{12}O_2$	164	0.02 ± 0.00	1.39 ± 0.03
31	47.933	1280	**Linalool oxide acetate[c]**	$C_{12}H_{20}O_3$	212	0.01 ± 0.00	0.57 ± 0.02
32	48.700	1291	1-H-Indol[d]	C_8H_7N	117	0.03 ± 0.00	2.16 ± 0.06
33	54.162	1372	Eugenol[a]	$C_{10}H_{12}O_2$	164	85.18 ± 0.15	5927.3 ± 16.72
34	55.675	1394	***β*-Elemene[d]**	$C_{15}H_{24}$	204	0.27 ± 0.00	18.59 ± 0.43
35	56.083	1400	Methyleugenol[d]	$C_{11}H_{14}O_2$	178	0.64 ± 0.01	44.58 ± 0.99
36	56.733	1410	***n*-Dodecanal[b]**	$C_{12}H_{24}O$	184	0.18 ± 0.00	12.39 ± 0.23
37	57.108	1416	***(Z)*-α- Bergamotene[d]**	$C_{15}H_{24}$	204	0.12 ± 0.00	8.00 ± 0.11
38	57.700	1426	*β*-Caryophyllene[d]	$C_{15}H_{24}$	204	1.74 ± 0.01	121.41 ± 1.35
39	58.533	1439	*α*-Guaiene[d]	$C_{15}H_{24}$	204	0.01 ± 0.00	0.57 ± 0.00
40	58.850	1444	Aromadendrene[d]	$C_{15}H_{24}$	204	0.01 ± 0.00	0.57 ± 0.01
41	59.175	1449	**Isogermacrene D[d]**	$C_{15}H_{24}$	204	0.12 ± 0.00	8.33 ± 0.18
42	59.933	1461	*α*-Humulene[d]	$C_{15}H_{24}$	204	0.87 ± 0.00	60.57 ± 0.55
43	60.050	1463	*(Z)*-α-Bisabilen[e]	$C_{15}H_{24}$	204	0.05 ± 0.00	3.76 ± 0.09
44	60.858	1475	**Cadina-1(6),4-diene[d]**	$C_{15}H_{24}$	204	0.02 ± 0.00	1.62 ± 0.11
45	61.142	1480	*γ*-Muurolene[d]	$C_{15}H_{24}$	204	4.49 ± 0.04	312.49 ± 4.42
46	61.208	1481	Valencene[d]	$C_{15}H_{24}$	204	0.22 ± 0.00	15.46 ± 0.23
47	61.325	1483	*α*-Curcumene[d]	$C_{15}H_{22}$	202	0.05 ± 0.00	3.65 ± 0.06
48	61.583	1487	***β*-*(Z)*-Bergamotene[d]**	$C_{15}H_{24}$	204	0.02 ± 0.00	1.08 ± 0.03
49	62.025	1494	*β*-Selinene[d]	$C_{15}H_{24}$	204	0.04 ± 0.00	2.94 ± 0.06
50	62.192	1496	***γ*-Amorphene[d]**	$C_{15}H_{24}$	204	0.04 ± 0.00	2.91 ± 0.03
51	62.367	1499	Cubebol[a]	$C_{15}H_{26}O$	222	0.18 ± 0.00	12.87 ± 0.35
52	62.483	1501	*α*-Muurolene[d]	$C_{15}H_{24}$	204	0.27 ± 0.00	18.66 ± 0.55
53	63.517	1518	Eugenyl acetate[c]	$C_{12}H_{14}O_3$	206	0.68 ± 0.01	47.29 ± 1.10
54	63.783	1523	*δ*-Cadinene[d]	$C_{15}H_{24}$	204	0.36 ± 0.00	24.87 ± 0.62
55	63.908	1525	***(E)*-Calamenene[d]**	$C_{15}H_{22}$	202	0.26 ± 0.00	17.77 ± 0.40
56	64.575	1536	***(E)*-Cadina-1,4-diene[d]**	$C_{15}H_{24}$	204	0.04 ± 0.00	2.49 ± 0.05
57	66.183	1563	**1-nor-Bourbonanone[e]**	$C_{14}H_{22}O$	206	0.03 ± 0.00	2.08 ± 0.07
58	67.692	1588	Caryophyllene oxide[f]	$C_{15}H_{24}O$	220	0.80 ± 0.23	55.96 ± 16.50
59	68.258	1597	**Salvial-4(14)-en-1-one[e]**	$C_{15}H_{24}O$	220	0.01 ± 0.01	0.97 ± 0.77
60	69.158	1613	**Tetradecanal[b]**	$C_{14}H_{28}O$	212	0.11 ± 0.00	7.56 ± 0.36
61	69.292	1615	**Humulene epoxide II[f]**	$C_{15}H_{24}O$	220	0.29 ± 0.00	20.01 ± 0.32
62	71.275	1650	*α*-Muurolol[a]	$C_{15}H_{26}O$	222	0.11 ± 0.00	7.63 ± 0.07
63	71.800	1659	**Cadin-4-en-10-ol[a]**	$C_{15}H_{26}O$	222	0.19 ± 0.00	13.23 ± 0.13
64	72.525	1672	***β*-Bisabolol[a]**	$C_{15}H_{26}O$	222	0.07 ± 0.00	5.17 ± 0.09
65	72.758	1676	Cadalene[d]	$C_{15}H_{18}$	198	0.06 ± 0.00	3.84 ± 0.04

Peak no	RT	RI	Compound name	MF	MW	Area (%)	Amount (mg/kg)
66	76.717	1748	*(E)*-Cadinol[a]	$C_{15}H_{26}O$	222	0.12 ± 0.00	8.17 ± 0.12
67	79.842	1806	**Farnesyl acetate[c]**	$C_{17}H_{28}O_2$	264	0.01 ± 0.00	0.62 ± 0.01
68	81.592	1840	**Phytone[e]**	$C_{18}H_{36}O$	268	0.04 ± 0.00	2.65 ± 0.06
69	85.083	1909	**Farnesyl acetone[e]**	$C_{18}H_{30}O$	262	0.03 ± 0.00	2.09 ± 0.01
70	86.858	1945	**Isophytol[a]**	$C_{20}H_{40}O$	296	0.01 ± 0.00	0.93 ± 0.02
			Total			**100.00**	**6958.51 ± 50.20**

Data were reported by mean ± standard deviation (n = 3); 0.00 = The value is less than 0.01; RT = Retention time in minutes; RI = Retention index; MF = Molecular formula; MW = Molecular weight; [a-k] Superscripts represent the functional group of compound (a = Alcohol; b = Aldehyde; c = Ester; d = Hydrocarbon; e = Ketone; f = Ether; g = Miscellaneous); I.S. = Internal standard; Bold mark compounds name = Firstly reported volatile compound in betel leaf; - = Not detected

Table 2.7. Relative content of functional group detected in *Piper betle* L. var. Sanchi from Bangladesh

Functional group	No	Area (%)	Amount (mg/kg)
Alcohol	13	86.88	6045.60
Aldehyde	11	0.64	44.34
Ester	9	0.96	66.63
Hydrocarbon	24	9.34	649.86
Ketone	5	0.12	8.47
Ether	7	2.03	141.45
Miscellaneous	1	0.03	2.16
Total	**70**	**100.00**	**6958.51**

No = No of volatile compounds; - = Not determined

Table 2.8. Relative content of terpene group detected in *Piper betle* L. var. Sanchi from Bangladesh

Terpene group	No	Area (%)	Amount (mg/kg)
Monoterpene hydrocarbons	4	0.29	20.29
Oxygenated monoterpene	7	85.74	5966.01
Sesquiterpene hydrocarbons	20	9.05	629.57
Oxygenated sesquiterpene	8	1.78	124.01
Oxygenated diterpene	1	0.01	0.93
Total	**40**	**96.87**	**6740.80**

No = No of volatile compounds

2.3.2.3. Volatile organic compounds in *Piper betle* L. var. Misti

The amount of volatile compounds in Misti betel leaf was 13958.90 mg/kg. There were identified 70 volatile compounds (Table 2.9), belongs to chemical classes of including 24 hydrocarbons (9.73 %, 1358.32 mg/kg), 19 alcohols (85.13 %, 11883.71 mg/kg), 11 esters (1.05 %, 146.84 mg/kg), 8 aldehydes (0.23 %, 32.14 mg/kg), 6 ethers (3.84 %, 536.03 mg/kg) and 2 ketones (0.01 %, 1.86 mg/kg) (Table 2.10). Terpene group (85.69 %, 11962.00 mg/kg) was the main class of volatile compounds in Misti betel leaf, where oxygenated monoterpene (74.07 %, 10339.53 mg/kg), sesquiterpene hydrocarbon (9.08 %, 1267.05 mg/kg), oxygenated sesquiterpene (1.87 %, 261.30 mg/kg), monoterpene hydrocarbon (0.65 %, 91.27 mg/kg) and oxygenated diterpene (0.02 %, 2.85 mg/kg) were quantified (Table 2.11). The major volatile compounds were eugenol (72.30 %, 10092.44 mg/kg), subsequently chavicol (11.95 %, 1667.43 mg/kg), *β*-caryophyllene (4.24 %, 592.42 mg/kg), valencene (3.02 %, 421.66 mg/kg) caryophyllene oxide (1.17 %, 162.95 mg/kg). Misti betel leaf has four different volatile compounds (pogostol, *α*-thujene, terpene-4-ol and dimethylallyl acetate) compared to others varieties (Table 2.18).

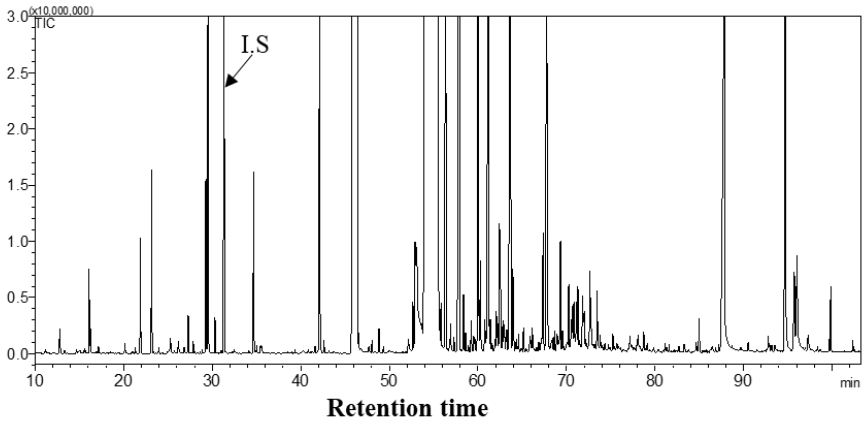

Figure 2.6. GC-MS chromatogram of volatile organic compounds in *Piper betle* L. var. Misti from Bangladesh, I.S. = Internal standard (*n*-butyl benzene).

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Table 2.9. Volatile compounds identified in *Piper betle* L. var. Misti from Bangladesh

Peak no	RT	RI	Compound name	MF	MW	Area (%)	Amount (mg/kg)
1	8.233	730	**2-Ethylfuran**[f]	C_6H_8O	96	0.06 ± 0.00	7.76 ± 0.08
2	11.200	776	**2-Penten-1-ol**[a]	$C_5H_{10}O$	86	0.00 ± 0.00	0.44 ± 0.01
3	12.725	799	**3-Hexenal**[b]	$C_6H_{10}O$	98	0.02 ± 0.00	2.19 ± 0.08
4	12.825	800	**Hexanal**[b]	$C_6H_{12}O$	100	0.03 ± 0.00	3.91 ± 0.05
5	14.683	828	Furfuraldehyde[b]	$C_5H_4O_2$	96	0.01 ± 0.00	0.84 ± 0.02
6	16.100	850	**2-Hexenal**[b]	$C_6H_{10}O$	98	0.1 ± 0.00	14.03 ± 0.17
7	16.225	852	3-Hexen-1-ol[a]	$C_6H_{12}O$	100	0.03 ± 0.00	4.56 ± 0.08
8	16.933	863	**2-Hexen-1-ol**[a]	$C_6H_{12}O$	100	0.00 ± 0.00	0.40 ± 0.01
9	17.167	866	**1-Hexanol**[a]	$C_6H_{14}O$	102	0.01 ± 0.00	1.19 ± 0.02
10	20.908	920	Dimethylallyl acetate[c]	$C_7H_{12}O_2$	128	0.00 ± 0.00	0.26 ± 0.01
11	21.333	925	*α*-Thujene[d]	$C_{10}H_{16}$	136	0.01 ± 0.00	1.07 ± 0.01
12	21.892	933	*α*-Pinene[d]	$C_{10}H_{16}$	136	0.15 ± 0.00	20.32 ± 0.15
13	23.158	950	Camphene[d]	$C_{10}H_{16}$	136	0.23 ± 0.00	32.68 ± 0.19
14	23.983	960	**Benzaldehyde**[b]	C_7H_6O	106	0.01 ± 0.00	0.91 ± 0.03
15	25.283	977	*β*-Pinene[d]	$C_{10}H_{16}$	136	0.02 ± 0.00	2.96 ± 0.07
16	25.733	983	Methyl heptenon[e]	$C_8H_{14}O$	126	0.00 ± 0.00	0.43 ± 0.01
17	27.300	1004	*(Z)*-3-Hexenyl-1-acetate[c]	$C_8H_{14}O_2$	142	0.05 ± 0.00	6.84 ± 0.11
18	27.860	1011	*n*-Hexyl acetate[c]	$C_6H_{16}O_2$	100	0.02 ± 0.00	2.18 ± 0.03
19	28.050	1014	*(E)*-2-Hexenyl acetate[c]	$C_8H_{14}O_2$	142	0.00 ± 0.00	0.33 ± 0.00
20	28.892	1025	*P*-cymene[d]	$C_{10}H_{14}$	134	0.00 ± 0.00	0.68 ± 0.01
21	29.275	1029	Limonene[d]	$C_{10}H_{16}$	136	0.24 ± 0.00	33.56 ± 0.19
22	29.517	1033	Eucalyptol[f]	$C_{10}H_{18}O$	154	0.73 ± 0.00	102.37 ± 0.70
23	30.317	1043	**Phenyl acetaldehyde**[b]	C_8H_8O	120	0.05 ± 0.00	6.64 ± 0.19
I.S.	31.350	1056	*n*-Butyl benzen[e]	C_9H_{14}	134	-	-
24	32.450	1071	*(Z)*-Sabinene hydrate[a]	$C_{10}H_{18}O$	154	0.00 ± 0.00	0.49 ± 0.01
25	34.675	1099	Linalool[a]	$C_{10}H_{18}O$	154	0.25 ± 0.00	34.76 ± 0.42
26	35.042	1104	**Nonanal**[b]	$C_9H_{18}O$	142	0.01 ± 0.00	1.17 ± 0.03
27	35.558	1111	**Phenylethyl alcohol**[a]	$C_8H_{10}O$	122	0.00 ± 0.00	0.37 ± 0.03
28	39.350	1162	Benzyl acetate[c]	$C_9H_{10}O_2$	150	0.00 ± 0.00	0.57 ± 0.12
29	40.858	1182	Terpinen-4-ol[a]	$C_{10}H_{18}O$	154	0.01 ± 0.00	0.79 ± 0.00
30	41.633	1192	Methyl salicylate[c]	$C_8H_8O_3$	152	0.01 ± 0.00	1.27 ± 0.01
31	41.917	1196	*α*-Terpineol[a]	$C_{10}H_{18}O$	154	0.00 ± 0.00	0.14 ± 0.00
32	42.075	1198	Estragole[f]	$C_{10}H_{12}O$	148	0.75 ± 0.01	104.39 ± 1.62
33	42.625	1206	Decanal[b]	$C_{10}H_{20}O$	156	0.02 ± 0.00	2.45 ± 0.03
34	45.758	1250	Chavicol[a]	$C_9H_{10}O$	134	11.95 ± 0.08	1667.43 ± 16.26

Peak no	RT	RI	Compound name	MF	MW	Area (%)	Amount (mg/kg)
35	46.050	1254	**2-Phenylethyl acetate[c]**	$C_{10}H_{12}O_2$	164	0.01 ± 0.00	1.71 ± 0.03
36	47.933	1280	**Linalool oxide acetate[c]**	$C_{12}H_{20}O_3$	212	0.02 ± 0.00	2.41 ± 0.03
37	49.958	1309	**4-vinyl Guaiacol[a]**	$C_9H_{10}O_2$	150	0.04 ± 0.03	6.18 ± 1.14
38	54.162	1372	Eugenol[a]	$C_{10}H_{12}O_2$	164	72.3 ± 0.22	10092.44 ± 0.23
39	55.675	1394	***β*-Elemene[d]**	$C_{15}H_{24}$	204	0.05 ± 0.00	7.46 ± 0.03
40	56.083	1400	Methyleugenol[d]	$C_{11}H_{14}O_2$	178	0.96 ± 0.00	133.93 ± 0.84
41	57.108	1416	***(Z)*-α- Bergamotene[d]**	$C_{15}H_{24}$	204	0.02 ± 0.00	2.66 ± 0.06
42	57.700	1426	*β*-Caryophyllene[d]	$C_{15}H_{24}$	204	4.24 ± 0.06	592.42 ± 10.53
43	58.533	1439	*α*-Guaiene[d]	$C_{15}H_{24}$	204	0.02 ± 0.00	3.42 ± 0.03
44	58.850	1444	Aromadendrene[d]	$C_{15}H_{24}$	204	0.01 ± 0.00	1.50 ± 0.02
45	59.175	1449	**Isogermacrene D[d]**	$C_{15}H_{24}$	204	0.05 ± 0.00	6.85 ± 0.08
46	59.933	1461	*α*-Humulene[d]	$C_{15}H_{24}$	204	0.95 ± 0.00	132.63 ± 1.13
47	60.858	1475	**Cadina-1(6),4-diene[d]**	$C_{15}H_{24}$	204	0.05 ± 0.00	6.46 ± 0.08
48	61.142	1480	*γ*-Muurolene[d]	$C_{15}H_{24}$	204	0.03 ± 0.00	3.50 ± 0.13
49	61.208	1481	Valencene[d]	$C_{15}H_{24}$	204	3.02 ± 0.04	421.66 ± 7.33
50	61.892	1492	**Eremophilene[d]**	$C_{15}H_{24}$	204	0.01 ± 0.00	1.55 ± 0.02
51	62.025	1494	*β*-Selinene[d]	$C_{15}H_{24}$	204	0.05 ± 0.00	7.64 ± 0.14
52	62.192	1496	***γ*-Amorphene[d]**	$C_{15}H_{24}$	204	0.03 ± 0.00	4.34 ± 0.01
53	62.367	1499	Cubebol[a]	$C_{15}H_{26}O$	222	0.24 ± 0.07	33.48 ± 10.9
54	62.508	1501	***α*-Amorphene[d]**	$C_{15}H_{24}$	204	0.13 ± 0.00	18.55 ± 0.78
55	63.517	1518	Eugenyl acetate[c]	$C_{12}H_{14}O_3$	206	0.92 ± 0.00	127.82 ± 0.19
56	63.783	1523	*δ*-Cadinene[d]	$C_{15}H_{24}$	204	0.2 ± 0.00	27.65 ± 1.19
57	63.908	1525	***(E)*-Calamenene[d]**	$C_{15}H_{22}$	202	0.14 ± 0.00	19.00 ± 0.02
58	64.575	1536	***(E)*-Cadina-1,4-diene[d]**	$C_{15}H_{24}$	204	0.02 ± 0.00	2.88 ± 0.02
59	66.142	1562	***α*-Nerolidol[a]**	$C_{15}H_{26}O$	222	0.03 ± 0.00	4.86 ± 0.07
60	67.692	1588	Caryophyllene oxide[f]	$C_{15}H_{24}O$	220	1.17 ± 0.01	162.95 ± 2.89
61	69.292	1615	**Humulene epoxide II[f]**	$C_{15}H_{24}O$	220	0.18 ± 0.00	24.63 ± 0.52
62	70.250	1632	Epicubenol[a]	$C_{15}H_{26}O$	222	0.09 ± 0.00	12.92 ± 0.21
63	71.275	1650	*α*-Muurolol[a]	$C_{15}H_{26}O$	222	0.05 ± 0.00	7.08 ± 0.06
64	71.958	1662	Pogostol[a]	$C_{15}H_{26}O$	222	0.07 ± 0.00	10.11 ± 0.10
65	72.758	1676	Cadalene[d]	$C_{15}H_{18}$	198	0.05 ± 0.00	6.89 ± 0.18
66	76.717	1748	*(E)*-Cadinol[a]	$C_{15}H_{26}O$	222	0.04 ± 0.00	5.26 ± 0.21
67	79.842	1806	**Farnesyl acetate[c]**	$C_{17}H_{28}O_2$	264	0.01 ± 0.00	1.42 ± 0.03
68	81.592	1840	**Phytone[e]**	$C_{18}H_{36}O$	268	0.01 ± 0.00	1.44 ± 0.05
69	86.858	1945	**Isophytol[a]**	$C_{20}H_{40}O$	296	0.01 ± 0.00	0.81 ± 0.03
70	99.658	2205	**Octadecanol acetate[c]**	$C_{20}H_{40}O_2$	312	0.01 ± 0.00	2.04 ± 0.04

Peak no	RT	RI	Compound name	MF	MW	Area (%)	Amount (mg/kg)
			Total			**100.00**	**13958.90 ± 60.10**

Data were reported by mean ± standard deviation (n = 3); 0.00 = The value is less than 0.01; RT = Retention time in minutes; RI = Retention index; MF = Molecular formula; MW = Molecular weight; [a-k] Superscripts represent the functional group of compound (a = Alcohol; b = Aldehyde; c = Ester; d = Hydrocarbon; e = Ketone; f = Ether; g = Miscellaneous); I.S. = Internal standard; Bold mark compounds name = Firstly reported volatile compound in betel leaf; - = Not detected

Table 2.10. Relative content of functional group detected in *Piper betle* L. var. Misti from Bangladesh

Functional group	No	Area (%)	Amount (mg/kg)
Alcohol	19	85.13	11883.71
Aldehyde	8	0.23	32.14
Ester	11	1.05	146.84
Hydrocarbon	24	9.73	1358.32
Ketone	2	0.01	1.86
Ether	6	3.84	536.03
Total	**70**	**100.00**	**13958.90**

No = No of volatile compounds; - = Not determined

Table 2.11. Relative content of terpene group detected in *Piper betle* L. var. Misti from Bangladesh

Terpene group	No	Area (%)	Amount (mg/kg)
Monoterpene	6	0.65	91.27
Oxygenated monoterpene	9	74.07	10339.53
Sesquiterpene hydrocarbons	18	9.08	1267.05
Oxygenated sesquiterpene	8	1.87	261.30
Oxygenated diterpene	2	0.02	2.85
Total	**43**	**85.69**	**11962.00**

No = No of volatile compounds

2.3.2.4. Volatile organic compounds in *Piper betle* L. var. Khasia

According to Table 2.12, the total of 74 volatile compounds were identified in Khasia betel leaf and their amount was 11109.70 mg/kg. For the better understanding, the chemical compounds were classified 27 hydrocarbons (19.49 %, 2165.88 mg/kg), 17 alcohols (76.39 %, 8486.81 mg/kg), 10 aldehydes (0.22 %, 24.82 mg/kg), 10 esters (0.49 %, 54.99 mg/kg), 7 ethers (3.34 %, 371.44 mg/kg) and 3 ketones (0.05 %, 5.72 mg/kg) (Table 2.13). Terpene group compound (92.66 %, 10294.39 mg/kg) was the vital class of volatile compounds in khasia betel leaf, where oxygenated monoterpene (70.44 %, 7825.41 mg/kg), sesquiterpene hydrocarbon (19.13 %, 2125.06 mg/kg), oxygenated sesquiterpene (2.72%, 302.14 mg/kg), monoterpene hydrocarbon (0.37 %, 40.82 mg/kg) and oxygenated diterpene (0.01 %, 0.96 mg/kg) were analysed (Table 2.14). The volatile compounds with the highest amount were eugenol (67.73 %, 7523.86 mg/kg) followed by valencene (6.18 %, 686.70 mg/kg), chavicol (6.08 %, 675.07 mg/kg), *β*-caryophyllene (4.93 %, 547.77 mg/kg), *δ*-cadenene (1.75 %, 194.36 mg/kg), *γ*-muurolene (1.74 %, 193.22 mg/kg), eucalyptol (1.32 %, 146.56 mg/kg), linalool (1.12 %, 123.96 mg/kg) and caryophyllene oxide (1.03 %, 114.33 mg/kg). Khasia variety has the highest number of uncommon compounds (dehydrocineole, 4-*δ*-carene, *γ*-terpinene, (*E*)-verbenol, *p*-cymen-8-ol, 4-allylphenyl acetate and *β*-sapthulenol) compare to other analysed betel leaves (Table 2.18).

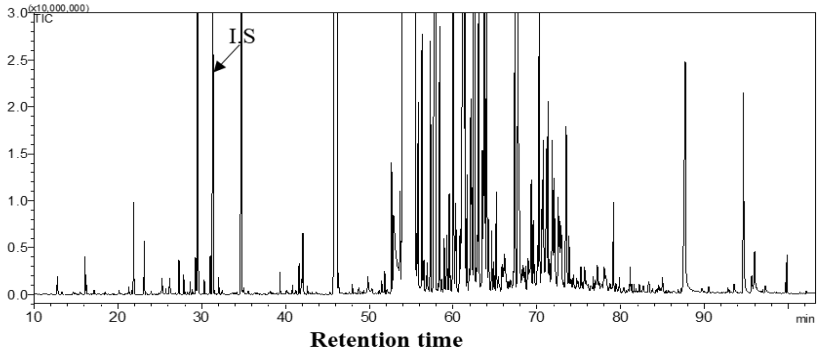

Figure 2.7. GC-MS chromatogram of volatile organic compounds in *Piper betle* L. var. Khasia from Bangladesh, I.S. = Internal standard (*n*-butyl benzene).

Table 2.12. Volatile compounds identified in *Piper betle* L. var. Khasia from Bangladesh

Peak no	RT	RI	Compound name	MF	MW	Area (%)	Amount (mg/kg)
1	8.233	730	**2-Ethylfuran**[f]	C_6H_8O	96	0.03 ± 0.00	2.79 ± 0.06
2	12.725	799	**3-Hexenal**[b]	$C_6H_{10}O$	98	0.01 ± 0.00	1.34 ± 0.25
3	12.825	800	**Hexanal**[b]	$C_6H_{12}O$	100	0.03 ± 0.00	3.12 ± 0.08
4	14.683	828	Furfuraldehyde[b]	$C_5H_4O_2$	96	0.00 ± 0.00	0.55 ± 0.01
5	16.100	850	**2-Hexenal**[b]	$C_6H_{10}O$	98	0.05 ± 0.00	6.06 ± 0.19
6	16.225	852	3-Hexen-1-ol[a]	$C_6H_{12}O$	100	0.01 ± 0.00	1.15 ± 0.04
7	17.167	866	**1-Hexanol**[a]	$C_6H_{14}O$	102	0.01 ± 0.00	0.66 ± 0.42
8	21.892	933	*α*-Pinene[d]	$C_{10}H_{16}$	136	0.16 ± 0.00	17.62 ± 0.66
9	23.158	950	Camphene[d]	$C_{10}H_{16}$	136	0.09 ± 0.00	10.37 ± 0.34
10	23.983	960	**Benzaldehyde**[b]	C_7H_6O	106	0.00 ± 0.00	0.55 ± 0.01
11	25.283	977	*β*-Pinene[d]	$C_{10}H_{16}$	136	0.03 ± 0.00	3.33 ± 0.29
12	25.733	983	Methyl heptenon[e]	$C_8H_{14}O$	126	0.01 ± 0.00	1.19 ± 0.04
13	26.208	990	Dehydrocineole[f]	$C_{10}H_{16}O$	152	0.04 ± 0.00	4.88 ± 0.20
14	27.300	1004	*(Z)*-3-Hexenyl-1-acetate[c]	$C_8H_{14}O_2$	142	0.06 ± 0.00	6.75 ± 0.27
15	27.860	1011	*n*-Hexyl acetate[c]	$C_6H_{16}O_2$	100	0.04 ± 0.00	3.92 ± 0.16
16	28.050	1014	*(E)*-2-Hexenyl acetate[c]	$C_8H_{14}O_2$	142	0.00 ± 0.00	0.42 ± 0.02
17	28.300	1017	4-*δ*-caren[e]	$C_{10}H_{16}$	136	0.00 ± 0.00	0.49 ± 0.01
18	28.892	1025	*P*-cymene[d]	$C_{10}H_{14}$	134	0.01 ± 0.00	1.01 ± 0.02
19	29.275	1029	Limonene[d]	$C_{10}H_{16}$	136	0.07 ± 0.00	7.39 ± 0.23
20	29.517	1033	Eucalyptol[f]	$C_{10}H_{18}O$	154	1.32 ± 0.01	146.56 ± 3.92
21	30.317	1043	**Phenyl acetaldehyde**[b]	C_8H_8O	120	0.03 ± 0.00	2.84 ± 0.09
22	31.033	1052	**2,3-Butanediyl diacetate**[c]	$C_8H_{14}O_4$	174	0.07 ± 0.00	7.43 ± 0.29
I.S.	31.350	1056	*n*-Butyl benzen[e]	C_9H_{14}	134	-	-
23	31.517	1058	*γ*-Terpinene[d]	$C_{10}H_{16}$	136	0.01 ± 0.00	0.61 ± 0.03
24	32.450	1071	*(Z)*-Sabinene hydrate[a]	$C_{10}H_{18}O$	154	0.01 ± 0.00	0.89 ± 0.04
25	34.675	1099	Linalool[a]	$C_{10}H_{18}O$	154	1.12 ± 0.02	123.96 ± 4.35
26	35.042	1104	**Nonanal**[b]	$C_9H_{18}O$	142	0.01 ± 0.00	0.86 ± 0.01
27	35.558	1111	**Phenylethyl alcohol**[a]	$C_8H_{10}O$	122	0.00 ± 0.00	0.17 ± 0.01
28	38.208	1146	***(E)*-Verbenol**[a]	$C_{10}H_{16}O$	152	0.01 ± 0.00	0.62 ± 0.05
29	39.350	1162	Benzyl acetate[c]	$C_9H_{10}O_2$	150	0.04 ± 0.00	4.81 ± 0.24
30	41.250	1187	***p*-Cymen-8-ol**[a]	$C_{10}H_{14}O$	150	0.01 ± 0.00	0.81 ± 0.04
31	41.633	1192	Methyl salicylate[c]	$C_8H_8O_3$	152	0.06 ± 0.00	6.63 ± 0.31
32	41.917	1196	*α*-Terpineol[a]	$C_{10}H_{18}O$	154	0.04 ± 0.00	4.62 ± 0.16
33	42.075	1198	Estragole[f]	$C_{10}H_{12}O$	148	0.13 ± 0.00	14.26 ± 0.78
34	42.625	1206	Decanal[b]	$C_{10}H_{20}O$	156	0.01 ± 0.00	1.60 ± 0.09
35	43.650	1220	***β*-Cyclocitral**[b]	$C_{10}H_{16}O$	152	0.01 ± 0.00	0.57 ± 0.03
36	45.758	1250	Chavicol[a]	$C_9H_{10}O$	134	6.08 ± 0.08	675.07 ± 18.74

Peak no	RT	RI	Compound name	MF	MW	Area (%)	Amount (mg/kg)
37	46.050	1254	**2-Phenylethyl acetate[c]**	$C_{10}H_{12}O_2$	164	0.02 ± 0.00	2.76 ± 0.11
38	52.125	1341	**4-Allylphenyl acetate[c]**	$C_{11}H_{12}O_2$	176	0.01 ± 0.00	1.41 ± 0.05
39	54.162	1372	Eugenol[a]	$C_{10}H_{12}O_2$	164	67.73 ± 0.63	7523.86 ± 42.04
40	55.675	1394	***β*-Elemene[d]**	$C_{15}H_{24}$	204	0.33 ± 0.00	36.21 ± 0.75
41	56.083	1400	Methyleugenol[d]	$C_{11}H_{14}O_2$	178	0.52 ± 0.02	57.67 ± 3.08
42	56.733	1410	***n*-Dodecanal[b]**	$C_{12}H_{24}O$	184	0.07 ± 0.00	7.33 ± 0.46
43	57.108	1416	***(Z)*-α-Bergamotene[d]**	$C_{15}H_{24}$	204	0.49 ± 0.01	54.48 ± 2.60
44	57.700	1426	*β*-Caryophyllene[d]	$C_{15}H_{24}$	204	4.93 ± 0.03	547.77 ± 11.24
45	58.225	1434	*γ*-Elemene[d]	$C_{15}H_{24}$	204	0.01 ± 0.00	1.28 ± 0.36
46	58.533	1439	*α*-Guaiene[d]	$C_{15}H_{24}$	204	0.57 ± 0.01	63.77 ± 2.67
47	58.850	1444	Aromadendrene[d]	$C_{15}H_{24}$	204	0.03 ± 0.00	2.97 ± 0.05
48	59.175	1449	**Isogermacrene D[d]**	$C_{15}H_{24}$	204	0.11 ± 0.00	12.22 ± 0.64
49	59.542	1455	**Sesquisabinene[d]**	$C_{15}H_{24}$	204	0.13 ± 0.00	14.86 ± 0.70
50	60.050	1463	*(Z)*-α-Bisabilen[e]	$C_{15}H_{24}$	204	0.21 ± 0.00	23.13 ± 1.21
51	61.142	1480	*γ*-Muurolene[d]	$C_{15}H_{24}$	204	1.74 ± 0.06	193.22 ± 9.38
52	61.208	1481	Valencene[d]	$C_{15}H_{24}$	204	6.18 ± 0.01	686.7 ± 8.43
53	61.325	1483	*α*-Curcumene[d]	$C_{15}H_{22}$	202	0.68 ± 0.00	75.59 ± 2.00
54	61.583	1487	***β*-*(Z)*-Bergamotene[d]**	$C_{15}H_{24}$	204	0.23 ± 0.00	25.61 ± 1.03
55	61.892	1492	**Eremophilene[d]**	$C_{15}H_{24}$	204	0.06 ± 0.00	6.61 ± 0.96
56	62.025	1494	*β*-Selinene[d]	$C_{15}H_{24}$	204	0.48 ± 0.04	53.3 ± 5.60
57	62.192	1496	***γ*-Amorphene[d]**	$C_{15}H_{24}$	204	0.21 ± 0.01	22.94 ± 1.98
58	63.783	1523	*δ*-Cadinene[d]	$C_{15}H_{24}$	204	1.75 ± 0.07	194.36 ± 10.93
59	63.908	1525	***(E)*-Calamenene[d]**	$C_{15}H_{22}$	202	0.66 ± 0.01	73.64 ± 2.39
60	64.575	1536	***(E)*-Cadina-1,4-diene[d]**	$C_{15}H_{24}$	204	0.12 ± 0.00	13.30 ± 0.69
61	66.142	1562	***α*-Nerolidol[a]**	$C_{15}H_{26}O$	222	0.14 ± 0.03	16.06 ± 4.40
62	67.692	1588	Caryophyllene oxide[f]	$C_{15}H_{24}O$	220	1.03 ± 0.04	114.33 ± 6.41
63	68.258	1597	**Salvial-4(14)-en-1-one[e]**	$C_{15}H_{24}O$	220	0.03 ± 0.00	2.84 ± 0.29
64	69.292	1615	**Humulene epoxide II[f]**	$C_{15}H_{24}O$	220	0.28 ± 0.01	30.94 ± 1.57
65	70.558	1637	*β*-Spathulenol[a]	$C_{15}H_{24}O$	220	0.15 ± 0.00	16.49 ± 1.16
66	71.275	1650	*α*-Muurolol[a]	$C_{15}H_{26}O$	222	0.36 ± 0.03	39.8 ± 3.99
67	71.800	1659	**Cadin-4-en-10-ol[a]**	$C_{15}H_{26}O$	222	0.34 ± 0.01	38.01 ± 2.40
68	72.525	1672	***β*-Bisabolol[a]**	$C_{15}H_{26}O$	222	0.37 ± 0.03	40.86 ± 4.53
69	72.758	1676	Cadalene[d]	$C_{15}H_{18}$	198	0.21 ± 0.00	23.12 ± 1.05
70	76.733	1744	**Guaiac acetate[c]**	$C_{17}H_{28}O_2$	264	0.14 ± 0.00	15.99 ± 1.23
71	76.717	1748	*(E)*-Cadinol[a]	$C_{15}H_{26}O$	222	0.03 ± 0.00	2.82 ± 0.16
72	79.842	1806	**Farnesyl acetate[c]**	$C_{17}H_{28}O_2$	264	0.04 ± 0.00	4.87 ± 0.32

Peak no	RT	RI	Compound name	MF	MW	Area (%)	Amount (mg/kg)
73	81.592	1840	**Phytone**[e]	$C_{18}H_{36}O$	268	0.02 ± 0.00	1.70 ± 0.11
74	86.858	1945	**Isophytol**[a]	$C_{20}H_{40}O$	296	0.01 ± 0.00	0.96 ± 0.10
			Total			**100.00**	**11109.70 ± 169.55**

Data were reported by mean ± standard deviation (n = 3); 0.00 = The value is less than 0.01; RT = Retention time in minutes; RI = Retention index; MF = Molecular formula; MW = Molecular weight; [a-k] Superscripts represent the functional group of compound (a = Alcohol; b = Aldehyde; c = Ester; d = Hydrocarbon; e = Ketone; f = Ether; g = Miscellaneous); I.S. = Internal standard; Bold mark compounds name = Firstly reported volatile compound in betel leaf; - = Not detected

Table 2.13. Relative content of functional group detected in *Piper betle* L. var. Khasia from Bangladesh

Functional group	No	Area (%)	Amount (mg/kg)
Alcohol	17	76.39	8486.81
Aldehyde	10	0.22	24.82
Ester	10	0.49	54.99
Hydrocarbon	27	19.49	2165.88
Ketone	3	0.05	5.72
Ether	7	3.34	371.44
Total	**74**	**100.00**	**11109.67**

No = No of volatile compounds; - = Not determined

Table 2.14. Relative content of terpene group detected in *Piper betle* L. var. Khasia from Bangladesh

Terpene group	No	Area (%)	Amount (mg/kg)
Monoterpene	7	0.37	40.82
Oxygenated monoterpene	12	70.44	7825.41
Sesquiterpene hydrocarbons	20	19.13	2125.06
Oxygenated sesquiterpene	9	2.72	302.14
Oxygenated diterpene	1	0.01	0.96
Total	**49**	**92.66**	**10294.39**

No = No of volatile compound

2.3.2.5. Volatile organic compounds in *Piper betle* L. var. BARI Paan 3

Analysis confirms that the yield of volatile compounds in BARI Paan 3 was 11684.10 mg/kg (dry weight basis) and there were 67 compounds belongs to chemical classes 22 hydrocarbons (11.16 %, 1303.74 mg/kg), 12 alcohols (86.41 %, 10096.81 mg/kg), 12 esters (0.45 %, 52.66 mg/kg), 10 aldehydes (0.45 %, 52.85 mg/kg), 7 ethers (1.48 %, 173.07 mg/kg) and 4 ketones (0.04 %, 4.96 mg/kg) (Table 2.15 and 2.16). Terpene group was the dominant chemical class with largest portion (94.62 %, 11055.59 mg/kg), while the oxygenated monoterpene were (82.13 %, 9595.97 mg/kg), sesquiterpene hydrocarbon (10.85 %, 1267.59 mg/kg), oxygenated sesquiterpene (1.31 %, 152.68 mg/kg), monoterpene hydrocarbon (0.31 %, 36.15 mg/kg) and oxygenated diterpene (0.03 %, 3.20 mg/kg) (Table 2.17). The main volatile compounds were eugenol (81.84 %, 9562.53 mg/kg) next to β-caryophyllene (4.69 %, 547.42 mg/kg), chavicol (3.97 %, 463.51 mg/kg), valencene (3.11 %, 362.85 mg/kg) and α-hemulene (1.03 %, 120.06 mg/kg). BARI Paan 3 has four compounds, which is not found in other varieties, including (*Z*)-2-pentenyl acetate, (*E*)-ocimene, *n*-decyl acetate and Undecane-2-one (Table 2.18).

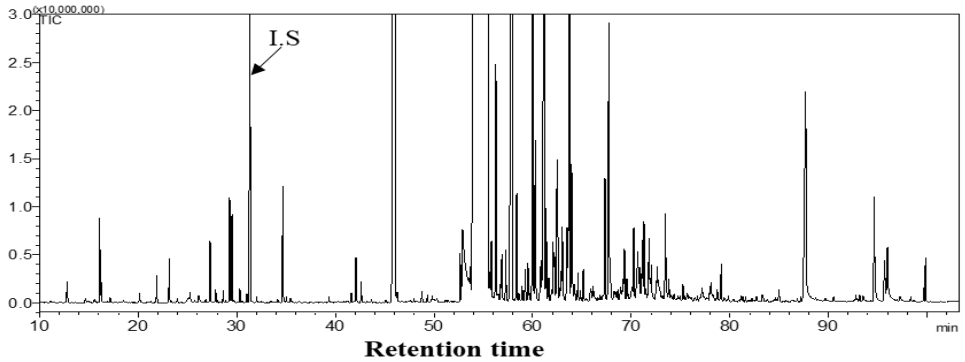

Figure 2.8. GC-MS chromatogram of volatile organic compounds in *Piper betle* L. var. BARI Pann 3 from Bangladesh, I.S. = Internal standard (*n*-butyl benzene).

Table 2.15. Volatile compounds identified in *Piper betle* L. var. BARI Paan 3 from Bangladesh

Peak no	RT	RI	Compound name	MF	MW	Area (%)	Amount (mg/kg)
1	8.233	730	**2-Ethylfuran**[f]	C_6H_8O	96	0.07 ± 0.00	8.50 ± 0.22
2	11.200	776	**2-Penten-1-ol**[a]	$C_5H_{10}O$	86	0.00 ± 0.00	0.22 ± 0.00
3	12.725	799	**3-Hexenal**[b]	$C_6H_{10}O$	98	0.02 ± 0.00	1.82 ± 0.12
4	12.825	800	**Hexanal**[b]	$C_6H_{12}O$	100	0.03 ± 0.00	3.58 ± 0.11
5	16.100	850	**2-Hexenal**[b]	$C_6H_{10}O$	98	0.13 ± 0.00	15.34 ± 0.36
6	16.225	852	3-Hexen-1-ol[a]	$C_6H_{12}O$	100	0.04 ± 0.00	4.16 ± 0.20
7	17.167	866	**1-Hexanol**[a]	$C_6H_{14}O$	102	0.01 ± 0.00	0.91 ± 0.05
8	20.150	910	***(Z)*-2-Pentenyl acetate**[c]	$C_7H_{12}O_2$	128	0.02 ± 0.00	1.81 ± 0.06
9	21.892	933	*α*-Pinene[d]	$C_{10}H_{16}$	136	0.04 ± 0.00	5.18 ± 0.10
10	23.158	950	Camphene[d]	$C_{10}H_{16}$	136	0.07 ± 0.00	8.64 ± 0.14
11	23.983	960	**Benzaldehyde**[b]	C_7H_6O	106	0.01 ± 0.00	0.69 ± 0.03
12	25.733	983	Methyl heptenon[e]	$C_8H_{14}O$	126	0.00 ± 0.00	0.49 ± 0.03
13	27.300	1004	*(Z)*-3-Hexenyl-1-acetate[c]	$C_8H_{14}O_2$	142	0.10 ± 0.00	11.92 ± 0.24
14	27.860	1011	*n*-Hexyl acetate[c]	$C_6H_{16}O_2$	100	0.02 ± 0.00	2.54 ± 0.08
15	28.050	1014	*(E)*-2-Hexenyl acetate[c]	$C_8H_{14}O_2$	142	0.00 ± 0.00	0.46 ± 0.02
16	28.892	1025	*P*-cymene[d]	$C_{10}H_{14}$	134	0.00 ± 0.00	0.45 ± 0.01
17	29.275	1029	Limonene[d]	$C_{10}H_{16}$	136	0.18 ± 0.00	21.58 ± 0.39
18	29.517	1033	Eucalyptol[f]	$C_{10}H_{18}O$	154	0.15 ± 0.00	17.55 ± 0.29
19	30.317	1043	**Phenyl acetaldehyde**[b]	C_8H_8O	120	0.03 ± 0.00	2.93 ± 0.11
20	30.558	1046	*(E)*-Ocimene[d]	$C_{10}H_{16}$	136	0.00 ± 0.00	0.29 ± 0.01
21	31.033	1052	**2,3-Butanediyl diacetate**[c]	$C_8H_{14}O_4$	174	0.01 ± 0.00	1.70 ± 0.05
I.S	31.350	1056	*n*-Butyl benzen[e]	C_9H_{14}	134	-	-
22	35.042	1104	**Nonanal**[b]	$C_9H_{18}O$	142	0.01 ± 0.00	0.95 ± 0.03
23	39.350	1162	Benzyl acetate[c]	$C_9H_{10}O_2$	150	0.01 ± 0.00	1.02 ± 0.04
24	41.633	1192	Methyl salicylate[c]	$C_8H_8O_3$	152	0.02 ± 0.00	2.08 ± 0.13
25	42.075	1198	Estragole[f]	$C_{10}H_{12}O$	148	0.08 ± 0.00	9.82 ± 0.18
26	42.625	1206	Decanal[b]	$C_{10}H_{20}O$	156	0.04 ± 0.00	4.15 ± 0.12
27	43.650	1220	***β*-Cyclocitral**[b]	$C_{10}H_{16}O$	152	0.01 ± 0.00	0.73 ± 0.00
28	45.758	1250	Chavicol[a]	$C_9H_{10}O$	134	3.97 ± 0.03	463.51 ± 6.50
29	46.050	1254	**2-Phenylethyl acetate**[c]	$C_{10}H_{12}O_2$	164	0.01 ± 0.00	1.18 ± 0.06
30	48.783	1292	**Undecan-2-one**[e]	$C_{11}H_{22}O$	170	0.02 ± 0.00	2.54 ± 0.04
31	54.162	1372	Eugenol[a]	$C_{10}H_{12}O_2$	164	81.84 ± 0.12	9562.53 ± 159.38
32	55.675	1394	***β*-Elemene**[d]	$C_{15}H_{24}$	204	0.09 ± 0.00	10.13 ± 0.17
33	56.083	1400	Methyleugenol[d]	$C_{11}H_{14}O_2$	178	0.43 ± 0.01	50.33 ± 0.82
34	56.758	1411	***n*-Decyl acetate**[c]	$C_{12}H_{24}O_2$	200	0.03 ± 0.00	3.95 ± 0.05
35	56.733	1410	***n*-Dodecanal**[b]	$C_{12}H_{24}O$	184	0.09 ± 0.00	10.56 ± 0.23
36	57.108	1416	***(Z)*-α- Bergamotene**[d]	$C_{15}H_{24}$	204	0.1 ± 0.00	11.14 ± 0.04
37	57.700	1426	*β*-Caryophyllene[d]	$C_{15}H_{24}$	204	4.69 ± 0.04	547.42 ± 3.04
38	58.533	1439	*α*-Guaiene[d]	$C_{15}H_{24}$	204	0.02 ± 0.00	1.77 ± 0.01
39	58.850	1444	Aromadendrene[d]	$C_{15}H_{24}$	204	0.03 ± 0.00	3.43 ± 0.05
40	59.175	1449	**Isogermacrene D**[d]	$C_{15}H_{24}$	204	0.07 ± 0.00	7.87 ± 0.09
41	59.542	1455	**Sesquisabinene**[d]	$C_{15}H_{24}$	204	0.06 ± 0.00	7.25 ± 0.12
42	59.933	1461	*α*-Humulene[d]	$C_{15}H_{24}$	204	1.03 ± 0.00	120.06 ± 1.87

Peak no	RT	RI	Compound name	MF	MW	Area (%)	Amount (mg/kg)
43	60.858	1475	**Cadina-1(6),4-diene**[d]	$C_{15}H_{24}$	204	0.06 ± 0.00	7.06 ± 0.14
44	61.142	1480	*γ*-Muurolene[d]	$C_{15}H_{24}$	204	0.07 ± 0.00	8.09 ± 0.14
45	61.208	1481	Valencene[d]	$C_{15}H_{24}$	204	3.11 ± 0.02	362.85 ± 4.72
46	61.325	1483	*α*-Curcumene[d]	$C_{15}H_{22}$	202	0.16 ± 0.00	18.56 ± 0.26
47	62.025	1494	*β*-Selinene[d]	$C_{15}H_{24}$	204	0.12 ± 0.00	14.11 ± 0.20
48	62.192	1496	***γ*-Amorphene**[d]	$C_{15}H_{24}$	204	0.09 ± 0.00	10.25 ± 0.25
49	62.508	1501	***α*-Amorphene**[d]	$C_{15}H_{24}$	204	0.45 ± 0.00	52.51 ± 0.72
50	63.517	1518	Eugenyl acetate[c]	$C_{12}H_{14}O_3$	206	0.19 ± 0.00	22.31 ± 0.23
51	63.908	1525	***(E)*-Calamenene**[d]	$C_{15}H_{22}$	202	0.68 ± 0.00	79.27 ± 1.45
52	64.575	1536	***(E)*-Cadina-1,4-diene**[d]	$C_{15}H_{24}$	204	0.05 ± 0.00	5.84 ± 0.10
53	66.142	1562	***α*-Nerolidol**[a]	$C_{15}H_{26}O$	222	0.02 ± 0.00	2.82 ± 0.03
54	67.692	1588	Caryophyllene oxide[f]	$C_{15}H_{24}O$	220	0.71 ± 0.00	82.8 ± 0.81
55	68.258	1597	**Salvial-4(14)-en-1-one**[e]	$C_{15}H_{24}O$	220	0.01 ± 0.00	0.98 ± 0.02
56	69.158	1613	**Tetradecanal**[b]	$C_{14}H_{28}O$	212	0.10 ± 0.00	12.1 ± 0.21
57	69.292	1615	**Humulene epoxide II**[f]	$C_{15}H_{24}O$	220	0.03 ± 0.00	4.06 ± 0.05
58	70.250	1632	Epicubenol[a]	$C_{15}H_{26}O$	222	0.13 ± 0.00	15.43 ± 0.06
59	71.275	1650	*α*-Muurolol[a]	$C_{15}H_{26}O$	222	0.11 ± 0.00	12.64 ± 0.11
60	71.800	1659	**Cadin-4-en-10-ol**[a]	$C_{15}H_{26}O$	222	0.12 ± 0.00	14.20 ± 0.17
61	72.525	1672	***β*-Bisabolol**[a]	$C_{15}H_{26}O$	222	0.14 ± 0.00	16.29 ± 0.21
62	76.717	1748	*(E)*-Cadinol[a]	$C_{15}H_{26}O$	222	0.03 ± 0.00	3.47 ± 0.02
63	79.842	1806	**Farnesyl acetate**[c]	$C_{17}H_{28}O_2$	264	0.01 ± 0.00	1.14 ± 0.03
64	81.592	1840	**Phytone**[e]	$C_{18}H_{36}O$	268	0.01 ± 0.00	0.95 ± 0.02
65	86.858	1945	**Isophytol**[a]	$C_{20}H_{40}O$	296	0.01 ± 0.00	0.65 ± 0.02
66	99.658	2205	**Octadecanol acetate**[c]	$C_{20}H_{40}O_2$	312	0.02 ± 0.00	2.55 ± 0.06
			Total			**100.00**	**11684.10 ± 185.62**

Data were reported by mean ± standard deviation (n = 3); 0.00 = The value is less than 0.01; RT = Retention time in minutes; RI = Retention index; MF = Molecular formula; MW = Molecular weight; [a-k] Superscripts represent the functional group of compound (a = Alcohol; b = Aldehyde; c = Ester; d = Hydrocarbon; e = Ketone; f = Ether; g = Miscellaneous); I.S. = Internal standard; Bold mark compounds name = Firstly reported volatile compound in betel leaf; - = Not detected

Table 2.16. Relative content of functional group detected in *Piper betle* L. var. BARI Paan 3 from Bangladesh

Functional group	No	Area (%)	Amount (mg/kg)
Alcohol	12	86.41	10096.81
Aldehyde	10	0.45	52.85
Ester	12	0.45	52.66
Hydrocarbon	22	11.16	1303.74
Ketone	4	0.04	4.96
Ether	7	1.48	173.07
Total	**67**	**100.00**	**11684.10**

No = No of volatile compounds; - = Not determined

Table 2.17. Relative content of terpene group detected in *Piper betle* L. var. BARI Paan 3 from Bangladesh

Terpene group	No	Area (%)	Amount (mg/kg)
Monoterpene	5	0.31	36.15
Oxygenated monoterpene	6	82.13	9595.97
Sesquiterpene hydrocarbons	17	10.85	1267.59
Oxygenated sesquiterpene	9	1.31	152.68
Oxygenated diterpene	2	0.03	3.20
Total	**39**	**94.62**	**11055.59**

No = No of compound

2.3.2.6. Comparison of volatile organic compound in FBL

The GC-MS chromatograms are shown in Figure 2.9. The identified volatile compound's details are listed in Table 2.18. The essential oil from *Piper betle* L. was found to contain a great variety of volatile compounds having a wide range of bioactivities. Generally, it is a mixture of 20-30 compounds, although 40-50 compounds can also be found (Basak and Guha, 2015). In this study, individually 67 to 74 volatile compounds were identified in FBL and this number is the highest number compared to other studies (Alighiri, et al., 2018; Karak et al., 2016). A total of 101 volatile compounds were identified in the analyzed betel leaves. To the best of our knowledge, 50 new betel leaf volatile compounds were reported for the first time in this analysis (Table 2.18, compounds name bold in mark). The total volatile compounds amount (dry weight basis) varied from 4346.84 mg/kg to 13958.90 mg/kg (Table 2.18).

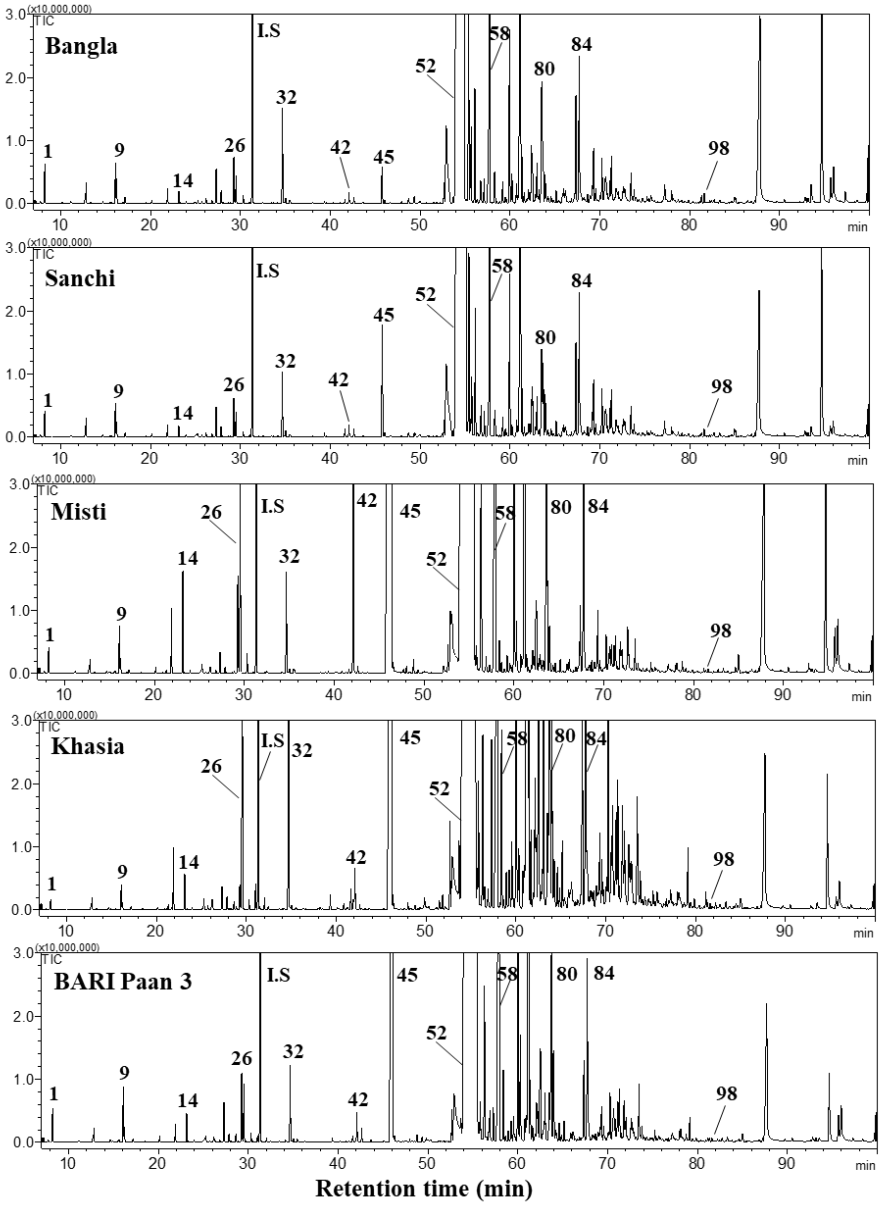

Figure 2.9. Comparison GC-MS chromatograms of volatile organic compounds in FBL from Bangladesh. Peak identification: I.S, Internal standard; 1, 2-Ethylfuran; 9, 1-Hexanol; 14, Camphene; 26, Eucalyptol; 32, Linalool; 42, Estragole; 45, Chavicol; 52, Eugenol; 58, *β*-Caryophyllene; 80, (*E*)-Calamenene; 84, Caryophyllene oxide; 98, Phyton.

Table 2.18. Comparison of **v**olatile compounds identified in FBL from Bangladesh

No	RT (Apx.)	RI (Apx.)	Compound name	MF	MW	Bangla		Sanchi		Misti		Khasia		BARI Paan 3	
						Area (%)	Amount (mg/kg)	Area (%)	Amount (mg/kg)	Area (%)	Amount (mg/kg)	Area (%)	Amount (mg/kg)	Area (%)	Amount (mg/kg)
1	8.233	730	**2-Ethylfuran**[f]	C_6H_8O	96	0.22 ± 0.00	9.41 ± 0.30	0.10 ± 0.00	7.17 ± 0.10	0.06 ± 0.00	7.76 ± 0.08	0.03 ± 0.00	2.79 ± 0.06	0.07 ± 0.00	8.50 ± 0.22
2	11.200	776	**2-Penten-1-ol**[a]	$C_5H_{10}O$	86	0.01 ± 0.00	0.27 ± 0.01	0.00 ± 0.00	0.23 ± 0.00	0.00 ± 0.00	0.44 ± 0.01	-	-	0.00 ± 0.00	0.22 ± 0.00
3	12.725	799	**3-Hexenal**[b]	$C_6H_{10}O$	98	0.05 ± 0.00	2.05 ± 0.05	0.01 ± 0.00	0.88 ± 0.08	0.02 ± 0.00	2.19 ± 0.08	0.01 ± 0.00	1.34 ± 0.25	0.02 ± 0.00	1.82 ± 0.12
4	12.825	800	**Hexanal**[b]	$C_6H_{12}O$	100	0.12 ± 0.00	5.12 ± 0.06	0.08 ± 0.00	5.67 ± 0.51	0.03 ± 0.00	3.91 ± 0.05	0.03 ± 0.00	3.12 ± 0.08	0.03 ± 0.00	3.58 ± 0.11
5	14.683	828	Furfuraldehyde[b]	$C_5H_4O_2$	96	0.01 ± 0.00	0.47 ± 0.01	0.01 ± 0.00	0.59 ± 0.01	0.01 ± 0.00	0.84 ± 0.02	0.00 ± 0.00	0.55 ± 0.01	-	-
6	16.100	850	**2-Hexenal**[b]	$C_6H_{10}O$	98	0.24 ± 0.00	10.29 ± 0.17	0.14 ± 0.00	0.00 ± 0.00	0.1 ± 0.00	14.03 ± 0.17	0.05 ± 0.00	6.06 ± 0.19	0.13 ± 0.00	15.34 ± 0.36
7	16.225	852	3-Hexen-1-ol[a]	$C_6H_{12}O$	100	0.15 ± 0.00	6.32 ± 0.10	0.06 ± 0.00	4.44 ± 0.07	0.03 ± 0.00	4.56 ± 0.08	0.01 ± 0.00	1.15 ± 0.04	0.04 ± 0.00	4.16 ± 0.20
8	16.933	863	**2-Hexen-1-ol**[a]	$C_6H_{12}O$	100	0.01 ± 0.00	0.44 ± 0.02	-	-	0.00 ± 0.00	0.40 ± 0.01	-	-	-	-
9	17.167	866	**1-Hexanol**[a]	$C_6H_{14}O$	102	0.04 ± 0.00	1.84 ± 0.03	0.02 ± 0.00	1.32 ± 0.02	0.01 ± 0.00	1.19 ± 0.02	0.01 ± 0.00	0.66 ± 0.42	0.01 ± 0.00	0.91 ± 0.05
10	20.150	910	***(Z)*-2-Pentenyl acetate**[c]	$C_7H_{12}O_2$	128	-	-	-	-	-	-	-	-	0.02 ± 0.00	1.81 ± 0.06
11	20.908	920	Dimethylallyl acetate[c]	$C_7H_{12}O_2$	128	-	-	-	-	0.00 ± 0.00	0.26 ± 0.01	-	-	-	-
12	21.333	925	*α*-Thujene[d]	$C_{10}H_{16}$	136	-	-	-	-	0.01 ± 0.00	1.07 ± 0.01	-	-	-	-
13	21.892	933	*α*-Pinene[d]	$C_{10}H_{16}$	136	0.09 ± 0.00	4.09 ± 0.05	0.05 ± 0.00	3.78 ± 0.05	0.15 ± 0.00	20.32 ± 0.15	0.16 ± 0.00	17.62 ± 0.66	0.04 ± 0.00	5.18 ± 0.10
14	23.158	950	Camphene[d]	$C_{10}H_{16}$	136	0.08 ± 0.00	3.56 ± 0.05	0.05 ± 0.00	3.45 ± 0.04	0.23 ± 0.00	32.68 ± 0.19	0.09 ± 0.00	10.37 ± 0.34	0.07 ± 0.00	8.64 ± 0.14
15	23.983	960	**Benzaldehyde**[b]	C_7H_6O	106	0.01 ± 0.00	0.49 ± 0.01	0.01 ± 0.00	0.43 ± 0.00	0.01 ± 0.00	0.91 ± 0.03	0.00 ± 0.00	0.55 ± 0.01	0.01 ± 0.00	0.69 ± 0.03
16	25.283	977	*β*-Pinene[d]	$C_{10}H_{16}$	136	0.02 ± 0.00	0.79 ± 0.01	0.01 ± 0.00	0.48 ± 0.01	0.02 ± 0.00	2.96 ± 0.07	0.03 ± 0.00	3.33 ± 0.29	-	-
17	25.733	983	Methyl heptenone[e]	$C_8H_{14}O$	126	0.01 ± 0.00	0.61 ± 0.01	0.01 ± 0.00	0.69 ± 0.01	0.00 ± 0.00	0.43 ± 0.01	0.01 ± 0.00	1.19 ± 0.04	0.00 ± 0.00	0.49 ± 0.03
18	26.192	989	**2-pentylfuran**[f]	$C_9H_{14}O$	138	0.04 ± 0.00	1.79 ± 0.02	0.02 ± 0.00	1.65 ± 0.02	-	-	-	-	-	-

No	RT (Apx.)	RI (Apx.)	Compound name	MF	MW	Bangla		Sanchi		Misti		Khasia		BARI Paan 3	
						Area (%)	Amount (mg/kg)	Area (%)	Amount (mg/kg)	Area (%)	Amount (mg/kg)	Area (%)	Amount (mg/kg)	Area (%)	Amount (mg/kg)
19	26.208	990	Dehydrocineole[f]	$C_{10}H_{16}O$	152	-	-	-	-	-	-	0.04 ± 0.00	4.88 ± 0.20	-	-
20	27.300	1004	*(Z)*-3-Hexenyl-1-acetate[c]	$C_8H_{14}O_2$	142	0.22 ± 0.00	9.50 ± 0.15	0.13 ± 0.00	9.25 ± 0.14	0.05 ± 0.00	6.84 ± 0.11	0.06 ± 0.00	6.75 ± 0.27	0.10 ± 0.00	11.92 ± 0.24
21	27.860	1011	*n*-Hexyl acetate[c]	$C_6H_{16}O_2$	100	0.09 ± 0.00	3.88 ± 0.07	0.05 ± 0.00	3.42 ± 0.04	0.02 ± 0.00	2.18 ± 0.03	0.04 ± 0.00	3.92 ± 0.16	0.02 ± 0.00	2.54 ± 0.08
22	28.050	1014	*(E)*-2-Hexenyl acetate[c]	$C_8H_{14}O_2$	142	0.01 ± 0.00	0.38 ± 0.01	0.01 ± 0.00	0.38 ± 0.01	0.00 ± 0.00	0.33 ± 0.00	0.00 ± 0.00	0.42 ± 0.02	0.00 ± 0.00	0.46 ± 0.02
23	28.300	1017	4-*δ*-caren[e]	$C_{10}H_{16}$	136	-	-	-	-	-	-	0.00 ± 0.00	0.49 ± 0.01	-	-
24	28.892	1025	*P*-cymene[d]	$C_{10}H_{14}$	134	0.01 ± 0.00	0.26 ± 0.01	-	-	0.00 ± 0.00	0.68 ± 0.01	0.01 ± 0.00	1.01 ± 0.02	0.00 ± 0.00	0.45 ± 0.01
25	29.275	1029	Limonene[d]	$C_{10}H_{16}$	136	0.31 ± 0.00	13.27 ± 0.16	0.18 ± 0.00	12.58 ± 0.15	0.24 ± 0.00	33.56 ± 0.19	0.07 ± 0.00	7.39 ± 0.23	0.18 ± 0.00	21.58 ± 0.39
26	29.517	1033	Eucalyptol[f]	$C_{10}H_{18}O$	154	0.18 ± 0.00	7.91 ± 0.10	0.11 ± 0.00	8.00 ± 0.11	0.73 ± 0.00	102.37 ± 0.70	1.32 ± 0.01	146.56 ± 3.92	0.15 ± 0.00	17.55 ± 0.29
27	30.317	1043	**Phenyl acetaldehyde[b]**	C_8H_8O	120	0.05 ± 0.00	2.39 ± 0.02	0.03 ± 0.00	1.82 ± 0.06	0.05 ± 0.00	6.64 ± 0.19	0.03 ± 0.00	2.84 ± 0.09	0.03 ± 0.00	2.93 ± 0.11
28	30.558	1046	*(E)*-Ocimene[g]	$C_{10}H_{16}$	136	-	-	-	-	-	-	-	-	0.00 ± 0.00	0.29 ± 0.01
29	31.033	1052	**2,3-Butanediyl diacetate[c]**	$C_8H_{14}O_4$	174	-	-	-	-	-	-	0.07 ± 0.00	7.43 ± 0.29	0.01 ± 0.00	1.70 ± 0.05
I.S.	31.350	1056	*n*-Butyl benzen[e]	C_9H_{14}	134	-	-	-	-	-	-	-	-	-	-
30	31.517	1058	*γ*-Terpinene[d]	$C_{10}H_{16}$	136	-	-	-	-	-	-	0.01 ± 0.00	0.61 ± 0.03	-	-
31	32.450	1071	*(Z)*-Sabinene hydrate[a]	$C_{10}H_{18}O$	154	-	-	-	-	0.00 ± 0.00	0.49 ± 0.01	0.01 ± 0.00	0.89 ± 0.04	-	-
32	34.675	1099	Linalool[a]	$C_{10}H_{18}O$	154	0.66 ± 0.01	28.53 ± 0.65	0.31 ± 0.00	21.9 ± 0.40	0.25 ± 0.00	34.76 ± 0.42	1.12 ± 0.02	123.96 ± 4.35	-	-
33	35.042	1104	**Nonanal[b]**	$C_9H_{18}O$	142	0.03 ± 0.00	1.40 ± 0.03	0.03 ± 0.00	1.86 ± 0.04	0.01 ± 0.00	1.17 ± 0.03	0.01 ± 0.00	0.86 ± 0.01	0.01 ± 0.00	0.95 ± 0.03
34	35.558	1111	**Phenylethyl alcohol[a]**	$C_8H_{10}O$	122	0.01 ± 0.00	0.38 ± 0.02	0.00 ± 0.00	0.24 ± 0.02	0.00 ± 0.00	0.37 ± 0.03	0.00 ± 0.00	0.17 ± 0.01	-	-
35	38.042	1144	**Oxophorone[e]**	$C_9H_{12}O_2$	152	0.01 ± 0.00	0.26 ± 0.01	-	-	-	-	-	-	-	-
36	38.208	1146	***(E)*-Verbenol[a]**	$C_{10}H_{16}O$	152	-	-	-	-	-	-	0.01 ± 0.00	0.62 ± 0.05	-	-
37	39.350	1162	Benzyl acetate[c]	$C_9H_{10}O_2$	150	0.01 ± 0.00	0.39 ± 0.01	0.02 ± 0.00	1.05 ± 0.01	0.00 ± 0.00	0.57 ± 0.12	0.04 ± 0.00	4.81 ± 0.24	0.01 ± 0.00	1.02 ± 0.04
38	40.858	1182	Terpinen-4-ol[a]	$C_{10}H_{18}O$	154	-	-	-	-	0.01 ± 0.00	0.79 ± 0.00	-	-	-	-

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						Area (%)	Amount (mg/kg)	Area (%)	Amount (mg/kg)	Area (%)	Amount (mg/kg)	Area (%)	Amount (mg/kg)	Area (%)	Amount (mg/kg)
39	41.250	1187	***p*-Cymen-8-ol**[a]	$C_{10}H_{14}O$	150	-	-	-	-	-	-	0.01 ± 0.00	0.81 ± 0.04	-	-
40	41.633	1192	Methyl salicylate[c]	$C_8H_8O_3$	152	0.03 ± 0.00	1.17 ± 0.02	0.04 ± 0.00	2.66 ± 0.01	0.01 ± 0.00	1.27 ± 0.01	0.06 ± 0.00	6.63 ± 0.31	0.02 ± 0.00	2.08 ± 0.13
41	41.917	1196	*α*-Terpineol[a]	$C_{10}H_{18}O$	154	-	-	-	-	0.00 ± 0.00	0.14 ± 0.00	0.04 ± 0.00	4.62 ± 0.16	-	-
42	42.075	1198	Estragole[f]	$C_{10}H_{12}O$	148	0.08 ± 0.00	3.34 ± 0.07	0.06 ± 0.00	4.08 ± 0.08	0.75 ± 0.01	104.39 ± 1.62	0.13 ± 0.00	14.26 ± 0.78	0.08 ± 0.00	9.82 ± 0.18
43	42.625	1206	Decanal[b]	$C_{10}H_{20}O$	156	0.04 ± 0.00	1.88 ± 0.06	0.04 ± 0.00	2.70 ± 0.06	0.02 ± 0.00	2.45 ± 0.03	0.01 ± 0.00	1.60 ± 0.09	0.04 ± 0.00	4.15 ± 0.12
44	43.650	1220	***β*-Cyclocitral**[b]	$C_{10}H_{16}O$	152	0.01 ± 0.00	0.51 ± 0.01	0.01 ± 0.00	0.65 ± 0.01	-	-	0.01 ± 0.00	0.57 ± 0.03	0.01 ± 0.00	0.73 ± 0.00
45	45.758	1250	Chavicol[a]	$C_9H_{10}O$	134	0.26 ± 0.00	11.39 ± 0.23	0.61 ± 0.01	42.18 ± 0.79	11.95 ± 0.08	1667.43 ± 16.26	6.08 ± 0.08	675.07 ± 18.74	3.97 ± 0.03	463.51 ± 6.50
46	46.050	1254	**2-Phenylethyl acetate**[c]	$C_{10}H_{12}O_2$	164	0.02 ± 0.00	0.97 ± 0.02	0.02 ± 0.00	1.39 ± 0.03	0.01 ± 0.00	1.71 ± 0.03	0.02 ± 0.00	2.76 ± 0.11	0.01 ± 0.00	1.18 ± 0.06
47	47.933	1280	**Linalool oxide acetate**[c]	$C_{12}H_{20}O_3$	212	0.05 ± 0.00	2.00 ± 0.05	0.01 ± 0.00	0.57 ± 0.02	0.02 ± 0.00	2.41 ± 0.03	-	-	-	-
48	48.700	1291	1-H-Indol[d]	C_8H_7N	117	-	-	0.03 ± 0.00	2.16 ± 0.06	-	-	-	-	-	-
49	48.783	1292	**Undecan-2-one**[e]	$C_{11}H_{22}O$	170	-	-	-	-	-	-	-	-	0.02 ± 0.00	2.54 ± 0.04
50	49.958	1309	**4-vinyl Guaiacol**[a]	$C_9H_{10}O_2$	150	0.01 ± 0.00	0.53 ± 0.01	-	-	0.04 ± 0.03	6.18 ± 1.14	-	-	-	-
51	52.125	1341	**4-Allylphenyl acetate**[c]	$C_{11}H_{12}O_2$	176	-	-	-	-	-	-	0.01 ± 0.00	1.41 ± 0.05	-	-
52	54.162	1372	Eugenol[a]	$C_{10}H_{12}O_2$	164	78.52 ± 0.27	3412.97 ± 1.31	85.18 ± 0.15	5927.3 ± 16.72	72.3 ± 0.22	10092.44 ± 0.23	67.73 ± 0.63	7523.86 ± 42.04	81.84 ± 0.12	9562.53 ± 159.38
53	55.675	1394	***β*-Elemene**[d]	$C_{15}H_{24}$	204	0.6 ± 0.01	26.24 ± 0.56	0.27 ± 0.00	18.59 ± 0.43	0.05 ± 0.00	7.46 ± 0.03	0.33 ± 0.00	36.21 ± 0.75	0.09 ± 0.00	10.13 ± 0.17
54	56.083	1400	Methyleugenol[d]	$C_{11}H_{14}O_2$	178	0.81 ± 0.01	35.18 ± 0.69	0.64 ± 0.01	44.58 ± 0.99	0.96 ± 0.00	133.93 ± 0.84	0.52 ± 0.02	57.67 ± 3.08	0.43 ± 0.01	50.33 ± 0.82
55	56.758	1411	***n*-Decyl acetate**[c]	$C_{12}H_{24}O_2$	200	-	-	-	-	-	-	-	-	0.03 ± 0.00	3.95 ± 0.05
56	56.733	1410	***n*-Dodecanal**[b]	$C_{12}H_{24}O$	184	0.19 ± 0.00	8.32 ± 0.20	0.18 ± 0.00	12.39 ± 0.23	-	-	0.07 ± 0.00	7.33 ± 0.46	0.09 ± 0.00	10.56 ± 0.23
57	57.108	1416	***(Z)*-α-Bergamotene**[d]	$C_{15}H_{24}$	204	0.17 ± 0.00	7.26 ± 0.18	0.12 ± 0.00	8.00 ± 0.11	0.02 ± 0.00	2.66 ± 0.06	0.49 ± 0.01	54.48 ± 2.60	0.1 ± 0.00	11.14 ± 0.04
58	57.700	1426	*β*-Caryophyllene[d]	$C_{15}H_{24}$	204	2.64 ± 0.04	114.69 ± 2.24	1.74 ± 0.01	121.41 ± 1.35	4.24 ± 0.06	592.42 ± 10.53	4.93 ± 0.03	547.77 ± 11.24	4.69 ± 0.04	547.42 ± 3.04
59	58.225	1434	γ-Elemene[d]	$C_{15}H_{24}$	204	-	-	-	-	-	-	0.01 ± 0.00	1.28 ± 0.36	-	-

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						Area (%)	Amount (mg/kg)	Area (%)	Amount (mg/kg)	Area (%)	Amount (mg/kg)	Area (%)	Amount (mg/kg)	Area (%)	Amount (mg/kg)
60	58.533	1439	*α*-Guaiene[d]	$C_{15}H_{24}$	204	0.01 ± 0.00	0.56 ± 0.01	0.01 ± 0.00	0.57 ± 0.00	0.02 ± 0.00	3.42 ± 0.03	0.57 ± 0.01	63.77 ± 2.67	0.02 ± 0.00	1.77 ± 0.01
61	58.850	1444	Aromadendrene[d]	$C_{15}H_{24}$	204	-	-	0.01 ± 0.00	0.57 ± 0.01	0.01 ± 0.00	1.50 ± 0.02	0.03 ± 0.00	2.97 ± 0.05	0.03 ± 0.00	3.43 ± 0.05
62	58.833	1445	**9-epi-β-Caryophyllene[d]**	$C_{15}H_{24}$	204	0.01 ± 0.00	0.51 ± 0.04	-	-	-	-	-	-	-	-
63	59.175	1449	**Isogermacrene D[d]**	$C_{15}H_{24}$	204	0.18 ± 0.00	7.94 ± 0.18	0.12 ± 0.00	8.33 ± 0.18	0.05 ± 0.00	6.85 ± 0.08	0.11 ± 0.00	12.22 ± 0.64	0.07 ± 0.00	7.87 ± 0.09
64	59.542	1455	**Sesquisabinene[d]**	$C_{15}H_{24}$	204	-	-	-	-	-	-	0.13 ± 0.00	14.86 ± 0.70	0.06 ± 0.00	7.25 ± 0.12
65	59.933	1461	*α*-Humulene[d]	$C_{15}H_{24}$	204	1.35 ± 0.02	58.77 ± 1.25	0.87 ± 0.00	60.57 ± 0.55	0.95 ± 0.00	132.63 ± 1.13	-	-	1.03 ± 0.00	120.06 ± 1.87
66	60.050	1463	*(Z)*-α-Bisabilen[e]	$C_{15}H_{24}$	204	-	-	0.05 ± 0.00	3.76 ± 0.09	-	-	0.21 ± 0.00	23.13 ± 1.21	-	-
67	60.858	1475	**Cadina-1(6),4-diene[d]**	$C_{15}H_{24}$	204	0.02 ± 0.00	0.69 ± 0.03	0.02 ± 0.00	1.62 ± 0.11	0.05 ± 0.00	6.46 ± 0.08	-	-	0.06 ± 0.00	7.06 ± 0.14
68	61.142	1480	*γ*-Muurolene[d]	$C_{15}H_{24}$	204	6.45 ± 0.10	280.36 ± 5.40	4.49 ± 0.04	312.49 ± 4.42	0.03 ± 0.00	3.50 ± 0.13	1.74 ± 0.06	193.22 ± 9.38	0.07 ± 0.00	8.09 ± 0.14
69	61.208	1481	Valencene[d]	$C_{15}H_{24}$	204	-	-	0.22 ± 0.00	15.46 ± 0.23	3.02 ± 0.04	421.66 ± 7.33	6.18 ± 0.01	686.7 ± 8.43	3.11 ± 0.02	362.85 ± 4.72
70	61.325	1483	*α*-Curcumene[d]	$C_{15}H_{22}$	202	0.31 ± 0.00	13.31 ± 0.36	0.05 ± 0.00	3.65 ± 0.06	-	-	0.68 ± 0.00	75.59 ± 2.00	0.16 ± 0.00	18.56 ± 0.26
71	61.583	1487	***β-(Z)*-Bergamotene[d]**	$C_{15}H_{24}$	204	0.09 ± 0.00	3.90 ± 0.10	0.02 ± 0.00	1.08 ± 0.03	-	-	0.23 ± 0.00	25.61 ± 1.03	-	-
72	61.892	1492	**Eremophilene[d]**	$C_{15}H_{24}$	204	0.02 ± 0.00	0.97 ± 0.04	-	-	0.01 ± 0.00	1.55 ± 0.02	0.06 ± 0.00	6.61 ± 0.96	-	-
73	62.025	1494	*β*-Selinene[d]	$C_{15}H_{24}$	204	0.07 ± 0.00	2.86 ± 0.03	0.04 ± 0.00	2.94 ± 0.06	0.05 ± 0.00	7.64 ± 0.14	0.48 ± 0.04	53.3 ± 5.60	0.12 ± 0.00	14.11 ± 0.20
74	62.192	1496	***γ*-Amorphene[d]**	$C_{15}H_{24}$	204	0.05 ± 0.00	1.98 ± 0.06	0.04 ± 0.00	2.91 ± 0.03	0.03 ± 0.00	4.34 ± 0.01	0.21 ± 0.01	22.94 ± 1.98	0.09 ± 0.00	10.25 ± 0.25
75	62.367	1499	Cubebol[a]	$C_{15}H_{26}O$	222	0.42 ± 0.01	18.44 ± 0.61	0.18 ± 0.00	12.87 ± 0.35	0.24 ± 0.07	33.48 ± 10.9	-	-	-	-
76	62.483	1501	*α*-Muurolene[d]	$C_{15}H_{24}$	204	0.36 ± 0.01	15.83 ± 0.53	0.27 ± 0.00	18.66 ± 0.55	-	-	-	-	-	-
77	62.508	1501	***α*-Amorphene[d]**	$C_{15}H_{24}$	204	-	-	-	-	0.13 ± 0.00	18.55 ± 0.78	-	-	0.45 ± 0.00	52.51 ± 0.72
78	63.517	1518	Eugenyl acetate[c]	$C_{12}H_{14}O_3$	206	1.50 ± 0.00	65.38 ± 0.60	0.68 ± 0.01	47.29 ± 1.10	0.92 ± 0.00	127.82 ± 0.19	-	-	0.19 ± 0.00	22.31 ± 0.23
79	63.783	1523	*δ*-Cadinene[d]	$C_{15}H_{24}$	204	0.35 ± 0.00	15.24 ± 0.37	0.36 ± 0.00	24.87 ± 0.62	0.2 ± 0.00	27.65 ± 1.19	1.75 ± 0.07	194.36 ± 10.93	-	-
80	63.908	1525	***(E)*-Calamenene[d]**	$C_{15}H_{22}$	202	0.24 ± 0.00	10.42 ± 0.06	0.26 ± 0.00	17.77 ± 0.40	0.14 ± 0.00	19.00 ± 0.02	0.66 ± 0.01	73.64 ± 2.39	0.68 ± 0.00	79.27 ± 1.45

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						Area (%)	Amount (mg/kg)	Area (%)	Amount (mg/kg)	Area (%)	Amount (mg/kg)	Area (%)	Amount (mg/kg)	Area (%)	Amount (mg/kg)
81	64.575	1536	**(*E*)-Cadina-1,4-diene**[d]	$C_{15}H_{24}$	204	0.03 ± 0.00	1.50 ± 0.03	0.04 ± 0.00	2.49 ± 0.05	0.02 ± 0.00	2.88 ± 0.02	0.12 ± 0.00	13.30 ± 0.69	0.05 ± 0.00	5.84 ± 0.10
82	66.142	1562	**α-Nerolidol**[a]	$C_{15}H_{26}O$	222	-	-	-	-	0.03 ± 0.00	4.86 ± 0.07	0.14 ± 0.03	16.06 ± 4 .40	0.02 ± 0.00	2.82 ± 0.03
83	66.183	1563	**1-nor-Bourbonanone**[e]	$C_{14}H_{22}O$	206	0.09 ± 0.00	4.05 ± 0.09	0.03 ± 0.00	2.08 ± 0.07	-	-	-	-	-	-
84	67.692	1588	Caryophyllene oxide[f]	$C_{15}H_{24}O$	220	1.34 ± 0.01	58.26 ± 0.97	0.80 ± 0.23	55.96 ± 16.50	1.17 ± 0.01	162.95 ± 2.89	1.03 ± 0.04	114.33 ± 6.41	0.71 ± 0.00	82.8 ± 0.81
85	68.258	1597	**Salvial-4(14)-en-1-one**[e]	$C_{15}H_{24}O$	220	0.03 ± 0.00	1.27 ± 0.06	0.01 ± 0.01	0.97 ± 0.77	-	-	0.03 ± 0.00	2.84 ± 0.29	0.01 ± 0.00	0.98 ± 0.02
86	69.158	1613	**Tetradecanal**[b]	$C_{14}H_{28}O$	212	0.11 ± 0.00	4.93 ± 0.08	0.11 ± 0.00	7.56 ± 0.36	-	-	-	-	0.10 ± 0.00	12.1 ± 0.21
87	69.292	1615	**Humulene epoxide II**[f]	$C_{15}H_{24}O$	220	0.39 ± 0.00	17.12 ± 0.35	0.29 ± 0.00	20.01 ± 0.32	0.18 ± 0.00	24.63 ± 0.52	0.28 ± 0.01	30.94 ± 1.57	0.03 ± 0.00	4.06 ± 0.05
88	70.250	1632	Epicubenol[a]	$C_{15}H_{26}O$	222	-	-	-	-	0.09 ± 0.00	12.92 ± 0.21	-	-	0.13 ± 0.00	15.43 ± 0.06
89	70.558	1637	*β*-Spathulenol[a]	$C_{15}H_{24}O$	220	-	-	-	-	-	-	0.15 ± 0.00	16.49 ± 1.16	-	-
90	71.275	1650	*α*-Muurolol[a]	$C_{15}H_{26}O$	222	0.02 ± 0.00	0.69 ± 0.04	0.11 ± 0.00	7.63 ± 0.07	0.05 ± 0.00	7.08 ± 0.06	0.36 ± 0.03	39.8 ± 3.99	0.11 ± 0.00	12.64 ± 0.11
91	71.800	1659	**Cadin-4-en-10-ol**[a]	$C_{15}H_{26}O$	222	0.12 ± 0.00	5.25 ± 0.09	0.19 ± 0.00	13.23 ± 0.13	-	-	0.34 ± 0.01	38.01 ± 2.40	0.12 ± 0.00	14.20 ± 0.17
92	71.958	1662	Pogostol[a]	$C_{15}H_{26}O$	222	-	-	-	-	0.07 ± 0.00	10.11 ± 0.10	-	-	-	-
93	72.525	1672	**β-Bisabolol**[a]	$C_{15}H_{26}O$	222	0.09 ± 0.00	4.07 ± 0.07	0.07 ± 0.00	5.17 ± 0.09	-	-	0.37 ± 0.03	40.86 ± 4.53	0.14 ± 0.00	16.29 ± 0.21
94	72.758	1676	Cadalene[d]	$C_{15}H_{18}$	198	0.01 ± 0.00	0.44 ± 0.03	0.06 ± 0.00	3.84 ± 0.04	0.05 ± 0.00	6.89 ± 0.18	0.21 ± 0.00	23.12 ± 1.05	-	-
95	76.733	1744	**Guaiac acetate**[c]	$C_{17}H_{28}O_2$	264	-	-	-	-	-	-	0.14 ± 0.00	15.99 ± 1.23	-	-
96	76.717	1748	*(E)*-Cadinol[a]	$C_{15}H_{26}O$	222	0.11 ± 0.01	4.75 ± 0.65	0.12 ± 0.00	8.17 ± 0.12	0.04 ± 0.00	5.26 ± 0.21	0.03 ± 0.00	2.82 ± 0.16	0.03 ± 0.00	3.47 ± 0.02
97	79.842	1806	**Farnesyl acetate**[c]	$C_{17}H_{28}O_2$	264	0.01 ± 0.00	0.37 ± 0.01	0.01 ± 0.00	0.62 ± 0.01	0.01 ± 0.00	1.42 ± 0.03	0.04 ± 0.00	4.87 ± 0.32	0.01 ± 0.00	1.14 ± 0.03
98	81.592	1840	**Phytone**[e]	$C_{18}H_{36}O$	268	0.07 ± 0.00	3.07 ± 0.06	0.04 ± 0.00	2.65 ± 0.06	0.01 ± 0.00	1.44 ± 0.05	0.02 ± 0.00	1.70 ± 0.11	0.01 ± 0.00	0.95 ± 0.02
99	85.083	1909	**Farnesyl acetone**[e]	$C_{18}H_{30}O$	262	0.02 ± 0.00	0.75 ± 0.03	0.03 ± 0.00	2.09 ± 0.01	-	-	-	-	-	-
100	86.858	1945	**Isophytol**[a]	$C_{20}H_{40}O$	296	0.02 ± 0.00	0.69 ± 0.01	0.01 ± 0.00	0.93 ± 0.02	0.01 ± 0.00	0.81 ± 0.03	0.01 ± 0.00	0.96 ± 0.10	0.01 ± 0.00	0.65 ± 0.02
101	99.658	2205	**Octadecanol acetate**[c]	$C_{20}H_{40}O_2$	312	-	-	-	-	0.01 ± 0.00	2.04 ± 0.04	-	-	0.02 ± 0.00	2.55 ± 0.06

No	RT (Apx.)	RI (Apx.)	Compound name	MF	MW	Bangla		Sanchi		Misti		Khasia		BARI Paan 3	
						Area (%)	Amount (mg/kg)	Area (%)	Amount (mg/kg)	Area (%)	Amount (mg/kg)	Area (%)	Amount (mg/kg)	Area (%)	Amount (mg/kg)
			Total			**100.00**	**4346.91 ± 20.03**	**100.00**	**6958.51 ± 50.20**	**100.00**	**13958.90 ± 60.10**	**100.00**	**11109.70 ± 169.55**	**100.00**	**11684.10 ± 185.62**

Data were reported by mean ± standard deviation (n = 3); 0.00 = The value is less than 0.01; RT = Retention time in minutes; RI = Retention index; MF = Molecular formula; MW = Molecular weight; $^{a\text{-}k}$ Superscripts represent the functional group of compound (a = Alcohol; b = Aldehyde; c = Ester; d = Hydrocarbon; e = Ketone; f = Ether; g = Miscellaneous); I.S. = Internal standard; Bold mark compounds name = Firstly reported volatile compound in betel leaf; Apx. = Approximately, - = Not detected

Table 2.19. Comparison of **r**elative content of functional group detected in FBL from Bangladesh

Functional group	Bangla			Sanchi			Misti			Khasia			BARI Paan-3		
	No	Area (%)	Amount (mg/kg)	No	Area (%)	Amount (mg/kg)	No	Area (%)	Amount (mg/kg)	No	Area (%)	Amount (mg/kg)	No	Area (%)	Amount (mg/kg)
Alcohol	15	80.44	3496.55	13	86.8	6045.60	19	85.13	11883.7	17	76.39	8486.81	12	86.41	10096.81
Aldehyde	11	0.87	37.82	11	0.64	44.34	8	0.23	32.14	10	0.22	24.82	10	0.45	52.85
Ester	9	1.93	84.02	9	0.96	66.63	11	1.05	146.84	10	0.49	54.99	12	0.45	52.66
Hydrocarbon	24	13.47	585.44	24	9.34	649.86	24	9.73	1358.32	27	19.49	2165.88	22	11.16	1303.74
Ketone	6	0.23	10.00	5	0.12	8.47	2	0.01	1.86	3	0.05	5.72	4	0.04	4.96
Ether	7	3.06	133.01	7	2.03	141.45	6	3.84	536.03	7	3.34	371.44	7	1.48	173.07
Miscellaneous	-	-	-	1	0.03	2.16	-	-	-	-	-	-	-	-	-
Total	**72**	**100.0**	**4346.91**	**70**	**100.0**	**6958.51**	**70**	**100.**	**13958.90**	**74**	**100.00**	**11109.70**	**67**	**100.00**	**11684.10**

No = No of volatile compounds; - = Not determined

Table 2.20. Comparison of relative content of terpene group detected in FBL from Bangladesh

Terpene group	Bangla			Sanchi			Misti			Khasia			BARI Paan-3		
	No	Area (%)	Amount (mg/kg)	No	Area (%)	Amount (mg/kg)	No	Area (%)	Amount (mg/kg)	No	Area (%)	Amount (mg/kg)	No	Area (%)	Amount (mg/kg)
Monoterpene hydrocarbons	5	0.51	21.96	4	0.29	20.29	6	0.65	91.27	7	0.37	40.82	5	0.31	36.15
Oxygenated monoterpene	7	79.51	3456.10	7	85.74	5966.01	9	74.07	10339.53	12	70.44	7825.41	6	82.13	9595.97
Sesquiterpene hydrocarbons	19	12.96	563.48	20	9.05	629.57	18	9.08	1267.05	20	19.13	2125.06	17	10.85	1267.59
Oxygenated sesquiterpene	8	2.53	109.84	8	1.78	124.01	8	1.87	261.30	9	2.72	302.14	9	1.31	152.68
Oxygenated diterpene	1	0.02	0.69	1	0.01	0.93	2	0.02	2.85	1	0.01	0.96	2	0.03	3.20
Total	**40**	**95.52**	**4152.07**	**40**	**96.87**	**6740.80**	**43**	**85.69**	**11962.00**	**49**	**92.66**	**10294.39**	**39**	**94.62**	**11055.59**

No = No of volatile compound

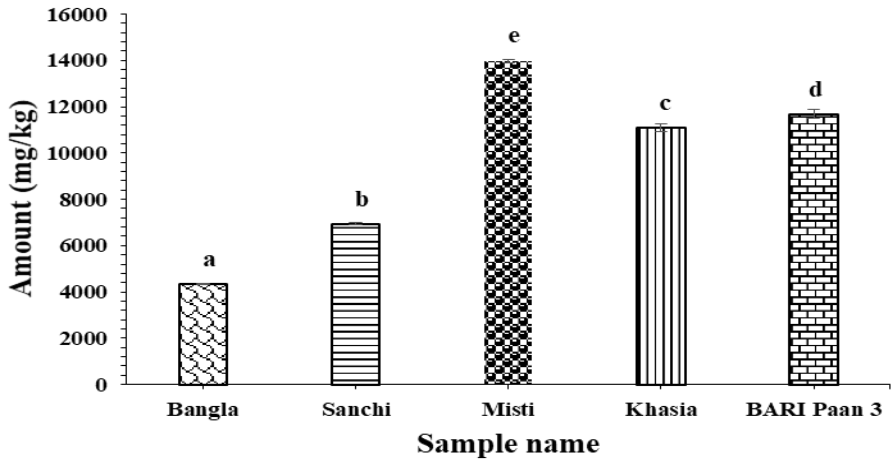


Figure 2.10. Comparison of the total volatile amount in FBL from Bangladesh. Lowercase letters a, b, c, and d on the bar diagram were used to mark the significance of difference ($p < 0.05$). Same letters between treatments mean insignificant difference. Different letters between treatments mean significant differences.

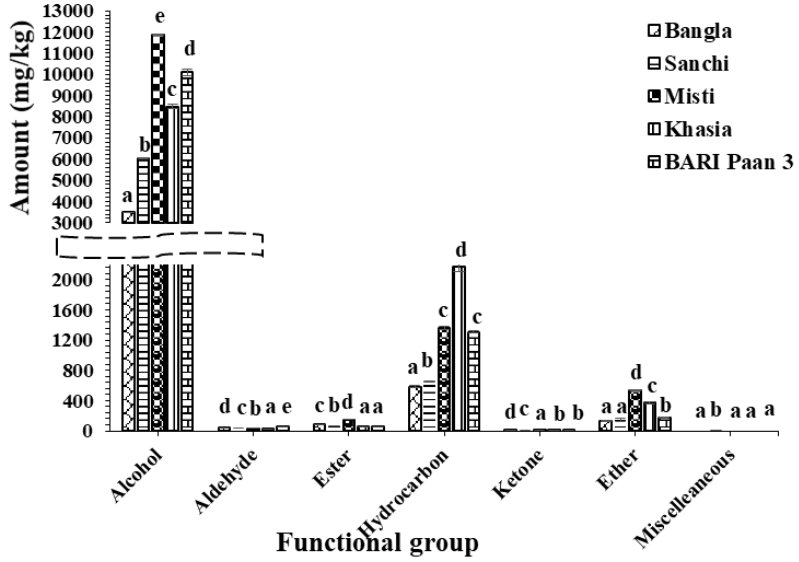


Figure 2.11. Comparison of functional group in FBL from Bangladesh. Lowercase letters a, b, c, d, and d on the bar diagram were used to mark the significance of difference ($p < 0.05$). Same letters between treatments mean insignificant difference. Different letters between treatments mean significant differences.

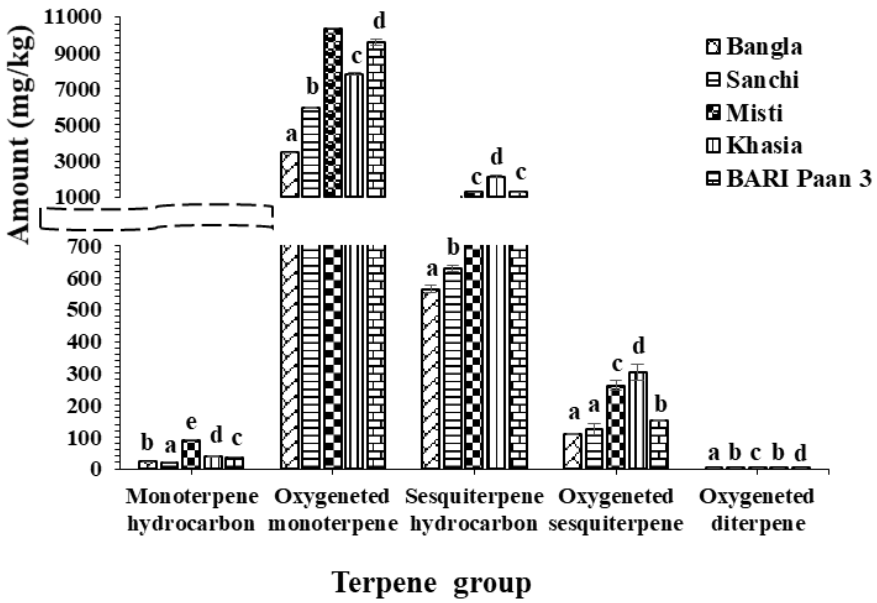


Figure 2.12. Comparison of terpene group in FBL from Bangladesh. Lowercase letters a, b, c, d, and d on the bar diagram were used to mark the significance of difference ($p < 0.05$). Same letters between treatments mean insignificant difference. Different letters between treatments mean significant differences.

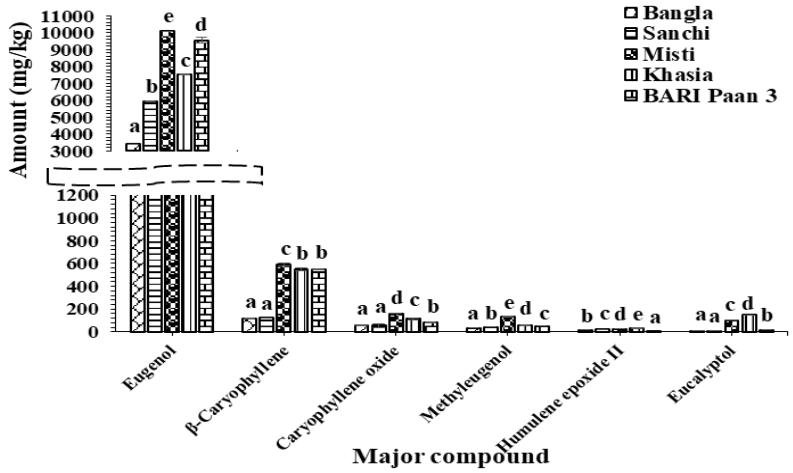


Figure 2.13. Comparison of common major volatile compounds in FBL from Bangladesh. Lowercase letters a, b, c, d, and d on the bar diagram were used to mark the significance of difference ($p < 0.05$). Same letters between treatments mean insignificant difference. Different letters between treatments mean significant differences.

Simultaneous distillation extraction (SDE) is widely known to be one of the prominent methods for extracting volatile compounds from different plant samples (Khan et al., 2015; Majcher et al., 2009). In this work, SDE was successfully applied to extract betel leaves volatile compounds and analyzed by GC-MS. The difference in the composition of volatile compounds was observed among betel leaf varieties. According to Table 2.18 and Table 2.19, it has been found that the alcohol group was the main functional group of compounds in betel leaves, where the phenolic compounds were the maximum. Additionally, the terpene group was the dominant chemical compound in FBL, where the oxygenated monoterpene-related compounds were the highest in amount (Table 2.19). Bajpai et al., 2010 have pointed out the major groups were terpene and phenol, which is similar to our observation. Earlier it has been described that SDE/GC-MS method is a suitable method for more volatile compound extraction and detection. Once again, it has been observed in this analysis.

As per Table 2.18, the eugenol was found in all varieties of betel leaf in high content, with peak area ranges from 67.73 % to 85.18 %, where Rawat et al., 1989 have reported that the eugenol peak area was 13.90 % to 63.56 % in betel leaves and they have used hydrodistillation method. Eugenol is widely used in agricultural applications to protect foods from microorganisms during storage and it has strong antimicrobial properties (Kamatou et al., 2012; Sugumaran et al., 2011). In our research, the eugenol content in Misti betel leaf has almost 3 and 2 times more than Bangla and Sanchi betel leaf, respectively, where Khasia and BARI Paan 3 betel leaf has nearly the same amount of eugenol. On the other hand, some studies of Indian and Srilankan betel leaf's major volatile constituents were isoeugenol, (*E*)-isoeugenol, acetyl eugenol, allylpyrocatechol and safrole (Kumar et al., 2007; Arambewela et al., 2005). The cultivating environment factor and betel leaf varieties should be responsible for this difference. The second major compounds are γ-muurolene (Bangla and Sanchi), β-caryophyllene (Misti and BARI Paan 3), and valencene (Khasia). The other major volatile compound details (eucalyptol, linalool,

estragole, chavicol, methyl eugenol, *α*-humulene, eugenyl acetate, *δ*-cadinene, (*Z*)-*α*-bisabolene, caryophyllene oxide and humulene epoxide II) are shown in Table 2.18.

Volatile compounds are responsible for aroma, taste, and bioactivity, and they are important for chemical fingerprinting. Chemical constituents of different varieties of plant essential oils are often used for the identification of variety or development of chemotypes. We have observed that betel leaves consist mostly of terpenoid and phenolic types of volatile compounds, which was an almost similar observation by other scientists (Guha and Nandi, 2019). Each country and possibly each region may have a distinct type of betel leaf essential oil called chemotypes. Different chemotypes such as chavicol, germacrene D, isoeugenol, chavibetol, eugenol, anethole, and safrole have been reported for most of the betel leaves essential oil (Kumar et al., 2007). This is mainly due to the ecological and geographical conditions, age of the plant, and time of harvesting. All these results confirm that analyzed betel leaves are eugenol chemotypes.

2.3.2.7. Multivariate statistical analysis in FBL

To determine the similarity and to classify the betel leaf varieties based on the identified volatile compounds, principal component analysis (PCA) and hierarchical cluster analysis (HCA) were applied. PCA was used to decrease the dimension of data variance, which was calculated based on the correlation of contents of volatile compounds. In PCA analysis, there are two eigenvalues higher than 1, which means that there could be two principal components for data analysis after the reduction dimension. However, the two principal components, i.e., PC1 (96.9%) and PC2 (2.2%) were used to simplify the statistical analysis and obtain a planer score plot of PCs. According to Figure 2.13, PC1 and PC2 can explain 99.1% of the data variance that means some information of the volatile compounds has been lost during the statistical re-modeling. Among the volatile compounds, eugenol and chavicol were the highest correlation based on loading of PC1 at 0.974 and 0.191, respectively. Oppositely, chavicol and valencene were the

maximum correlation based on loading of PC2 at 0.947 and 0.139, individually. The analyzed betel leaves formed into three clusters in the score plot of principal component analysis (Figure 2.13): Misti and BARI Paan 3 formed cluster I; Bangla formed cluster II; and Sanchi and Khasia formed cluster III.

We performed a hierarchical cluster analysis based on the amount of the 101 volatile compounds. We used Ward's method for between-group linkage and the similarity between clusters as a proximate analysis. The FBL formed three clusters in the dendrogram (Figure 2.15): Misti and BARI Paan 3 betel leaves for cluster I; Bangla betel leaf for cluster II; and Sanchi and Khasia betel leaves for cluster III, respectively. Cluster analysis can obtain a variety of classification results according to the scale.

PCA is a method that can be used to identify patterns in a data set and to reduce the dimensionality of multivariate data by removing inter-correlation among variables (Kim et al., 2014). The plant varieties could be separated into different clusters when analyzed by PCA, HCA, PLS-DA (partial least square- discriminant analysis) and/or other multivariate analysis on the basis of the different constituents. Karak et al. (2016) reported that the betel leaf varieties Meetha (Misti) and Chhaanchi (Sanchi) were distinctly different from the other analyzed varieties of betel leaf and it was evaluated by PCA and PLS-DA method. The same observation was found in our study, whereas Misti and Sanchi betel leaf varieties were observed in a different cluster from other analyzed betel leaf varieties and PCA and HCA performed this analysis.

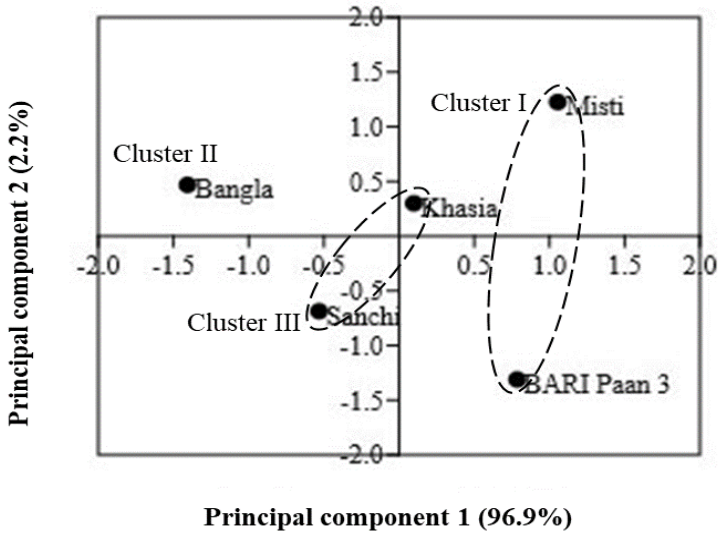


Figure 2.14. Score plot of first two principal components analysis based on volatile organic compound in FBL from Bangladesh.

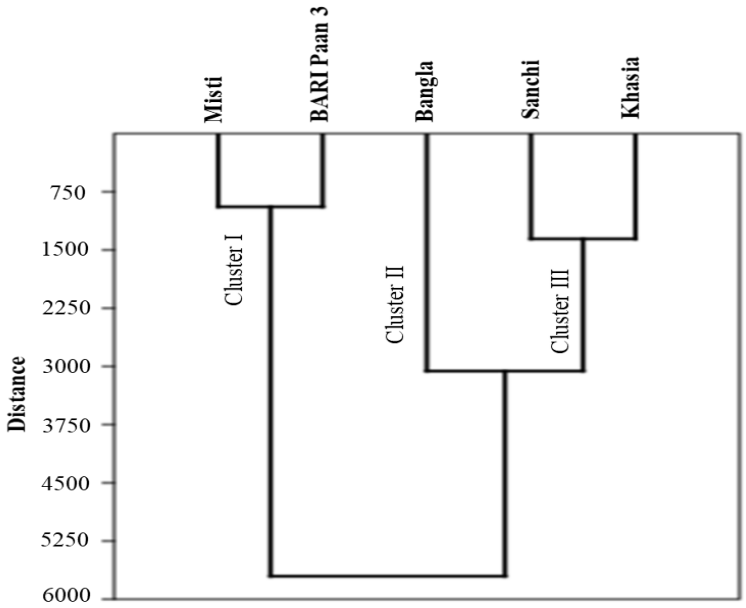


Figure 2.15. Dendrogram from volatile compounds in FBL from Bangladesh.

2.4. Conclusion

Simultaneous distillation extraction with *n*-pentane and diethyl ether solvent was used to extract volatile compounds from FBL, which resulted in 50 more volatile compounds compared to published literature. GC-MS analysis showed remarkable differences in the amount of volatile compounds among target leaves of betel varieties. The highest amount of volatile compounds was found in Misti betel leaf (13958.90 mg/kg) while the lowest amount was found in Bangla betel leaf (4346.91 mg/kg). Eugenol was present with the highest amount in all varieties with the peak area varying from 67.73 % to 85.18 %. The other major volatile compounds found across all varieties of betel leaves were β-caryophyllene, valencene, γ-muurolene, chavicol, and caryophyllene oxide. Through the PCA and HCA analysis, 5 varieties of betel leaf were separated and classified into three clusters based on their volatile compounds; identified in the current study. The finding of this research provide the expanded knowledge of volatile compounds in betel leaves, which could help the food, cosmetic, and medicinal industries to select more suitable *Piper betle* L. cultivars for volatile compound extraction.

CHAPTER III

Non-volatile organic compound screening, optimization of ultrasonic methods for the extraction of total phenol, and HPLC-DAD analysis in five varieties of *Piper betle* L. leaves (FBL) from Bangladesh

3.1. Introduction

Piper betle L. leaves are considered an economically and environmentally significant species in the family Piperaceae exist in of about 1200-2000 species. Betel leaves are very nutritious and contain large amounts of vitamins, minerals, volatile organic compounds, and non-volatile organic compounds (Fazal et al., 2014). The study of the polyphenolic and flavonoid composition in medicinal plants is the great interest to knowing the qualitative and quantitative difference (Meng et al., 2009). Most earlier betel leaf studies were based on the components present and this amount depending on varieties, harvesting time, and environmental condition (Dalai et al., 2014; Nouri & Nafchi, 2014; Paranjpe et al., 2013). The previous research is focused on the components present in betel leaf extracts but did not more studied about ultrasonic method optimization to increase the major phenolic compound extraction (Dalai et al., 2014; Nouri and Nafchi, 2014). This chapter focuses on optimizing the ultrasonic extraction method and quantifying total phenol, total flavonoid and HPLC-DAD analysis to quantify major phenolic compound hydroxychavicol in FBL from Bangladesh.

3.1.1. Importance of non-volatile organic compounds in *Piper betle* L. leaves

The non-nutritive bioactive compounds produced by plants through multiple metabolic pathways are called phytochemicals, which are volatile or non-volatile organic compounds. These are non-volatile compounds used by plants for self-defense and survival (Mota et al., 2009). The use of the herb as medicine is continuously expanding worldwide and natural products are

being used as potential sources of new bioactive molecules in the pharmaceutical industries (Mota et al., 2009). The therapeutic effects of betel leaf are primarily attributable to the existence of different groups of non-volatile compounds such as phenol, flavonoids, saponin, phenolic acids, antioxidant micronutrients, and etc (Tiara and Farida, 2013). It was documented that these non-volatile compounds have antioxidant, antimicrobial, anti-carcinogenic, anti-inflammatory, and many other properties (Srinivasan, 2005; Lampe, 2003). The non-volatile organic compound, especially the phenolic compound extract method, optimizes their major compound is very important for pharmaceutical industries. There are no ultrasonic method optimization reports, followed by quantifying their total phenol, total flavonoid, and major phenolic compound in FBL from Bangladesh. It is very important to determine a suitable extraction technique that extracts the non-volatile organic compound from betel leaves. Solvent selection is an important factor in any extraction. Generally, methanol, ethanol, propanol, acetone, and ethyl acetate, are commonly used for the extraction of phenolics from medicinal plants or fresh products (Durling et al., 2007). The suitable extraction method and the optimization of the extraction technique parameters improve the essential order to extract more phytochemicals for further processing in pharmaceutical industries.

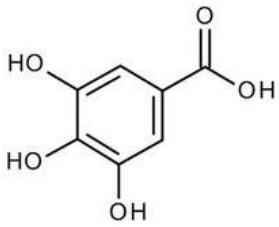
3.1.1.1. Phenolic compound

The term phenolic compounds encompass more than 4000 various compounds that contain an aromatic ring with one or more OH groups. The phenolic compounds are the major class of secondary metabolites that are broadly distributed in plants. These compounds have to contain one aromatic ring (cinnamic acid and gallic acid) and complex polymeric compounds (lignin, tannin, and etc.) (Abdou et al., 2010). The significant subclass of phenolic compounds includes phenol, phenolic acid, flavonoids, lignin, isoflavonoids, benzophenones, chromones, coumarin, and xanthones (Table 3.1). These polyphenols play a vital role in treating and preventing diabetics, cancer, cardiovascular disease, asthma, alzheimer disease, and etc (Scalbert

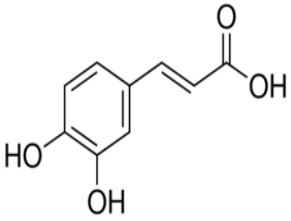
et al., 2005). Researchers are currently interested in preventing and treating diseases by simply improving the dietary intake of nutrients with antioxidant properties such as vitamin E, vitamin C, phenolic compounds, flavonoids, stilbene, anthocyanin, tannis, and etc. Therefore the interest is increasing in betel leaves chewing as potential anti-oxidative sources.

Table 3.1. Classification of phenolic compounds and their general structure

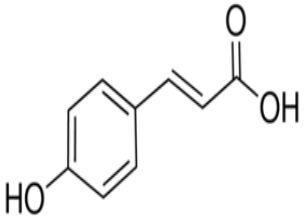
SL. No.	Class	Number of carbon atoms	Basis structure
1	Simple phenolics	6	C_6
2	Phenolic acids	7	$C_6 – C_8$
3	Stilbenes	14	$C_6 – C_2 – C_6$
4	Flavonoids	15	$C_6 – C_3 – C_6$
5	Lignans	18	$(C_6 – C_3)_2$



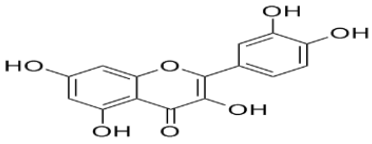
Gallic acid



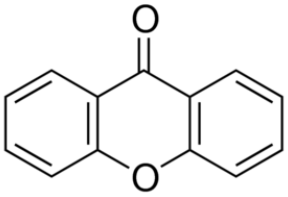
Caffeic acid



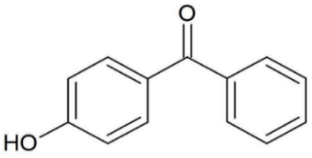
Cumaric acid



Quercetin



Xanthone

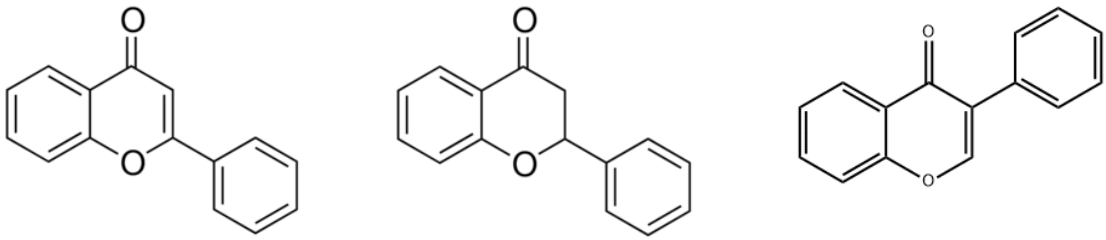


Benzophenone

Figure 3.1. Some important phenolic compound structure.

3.1.1.2. Flavonoid compound

Flavonoid is a very important class of polyphenolic compounds for the human diet, which may occur both in combined (glycones) forms and free (aglycones) forms, with a difference in the position number substitution and in unsaturation. It is classified as flavonols, flavanones, flavones, isoflavones, and anthocyanidin. Flavonoid plays important roles in the complications of scavenging free radicals, gastrointestinal tract, antiplatelet aggregator, antiulcer, antidiarrheal, antibacterial, antioxidant, antiviral, and anti-inflammatory agents (Lin et al., 2008; Arct and Pytkowska, 2008). The potentiality depends on the various mechanisms of flavonoids for example, inhibit cyclooxygenase (prostaglandins), block the angiotensin altering enzymes (high blood pressure), and block the estrogen generating enzymes (estrogen related cancers) (Dillard and German, 2000).



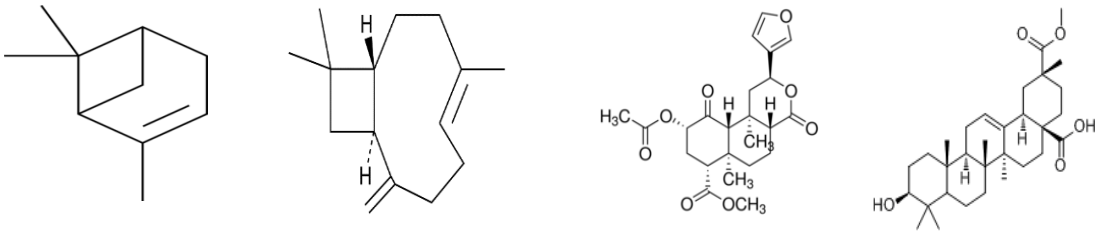
Flavone Flavanone Isoflavone

Figure 3.2. Some important flavonoid compound structure.

3.1.1.3. Terpene compound

Terpene is the other largest phytochemicals such as monoterpenoids, sesquiterpenoids, diterpenoids, triterpenoids saponins, saponins glycosides, phytosterols, and carotenoids. These are found in fruits, grains, and green vegetables. Normally, plants use triterpenes for carbon fixation through photosynthesis and get protected from diseases involving growth dysregulation and chronic damage. Animals use these phytochemicals for growth regulatory and hormonal

functions. They are reported to possess antimicrobial (Viuda-Martos et al., 2010), antioxidant (Milan et al., 2008), and antiviral properties (Orhan et al., 2012).

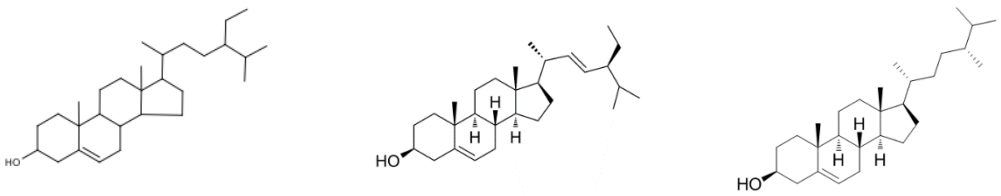


α-pinene Caryophyllene Salvinorin A Serjanic acid

Figure 3.3. Some important terpene compound structure.

3.1.1.4. Phytosterol compound

Oil and vegetable are the richest sources of phytochemicals naturally. They exist in free form or as cinnamic acid, ester of fatty acid, and glycosides. Campesterol, stigmasterol, *β*-sitosterol, were 30%, 3%, 65% of diet contents, respectively. These are the maximum commonly occurring phytosterols in the human diet (Weihrauch and Gardner, 1978). Phytosterols play important pharmacological roles in animals, such as anti-neoplastic, antipyretic, anti-inflammatory, and anti-immuno-modulatory activities.

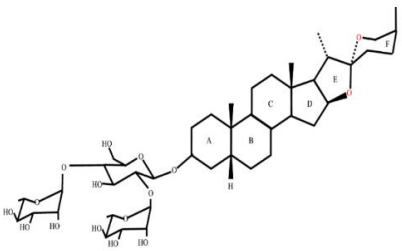


β-sitosterol Stigmasterol Campesterol

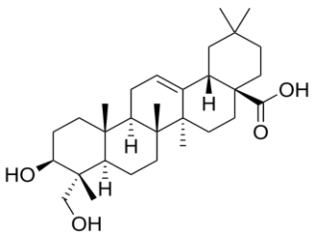
Figure 3.4. Some important phytosterol compound structure.

3.1.1.5. Saponins compound

Saponins are a different group of chemical compounds broadly distributed in the plant kingdom. These are characterized by their chemical structure containing a steroid aglycone or triterpene and attached one or more sugar chains. Consumer demand for natural products and their physicochemical properties and mounting indication on their biological activity for example, anti-cholesterol and anticancer activity. Saponins as commercially important chemical compounds and their application increase in the cosmetics, pharmaceutical, and food sectors. The understanding of their full commercial potential needs to develop new processing strategies.



Steroidal saponins



Hederagenin

Figure 3.5. Some important saponin compound structure.

3.1.2. Method of extraction

Extraction method choice is an important factor for extracting bioactive compounds from medicinal plants. Effective, low-cost, energy-efficient, and low-temperature extraction systems must be built to utilize bioactive compounds for beneficial properties. Phenolic compound extraction occurs in four phases: pre-treatment, extraction, purification/isolation, and encapsulation (Muhamad et al., 2017). There are many pre-treatment methods, such as maceration, milling, grinding, drying, and homogenization (Routray et al., 2012). Homogenization breaks down the sample cellular structure and increases the contact surface

region, bioavailability, and shelf life of phenolic compounds (DiNardo et al., 2019). Based on the plant material, extraction methods are selected for extracting the phenolic compound.

3.1.2.1. Heat reflux extraction

The traditional extraction method of heat reflux is used to extract the bioactive compound from a medicinal plant with heat and agitation using solvents such as methane, hexane, methanol, and ethanol (Bandar et al., 2013). For the extraction of phenolic compounds, heat reflux extraction is frequently used as it is an easy and cheap method. The process of extraction is carried out by mass transfer and involves several steps to complete the transfer of the solution into solvent: i) The penetration of the solvent into the solid matrix, ii) The solubilization of the components iii) The transfer of the solvent to the solid matrix and iv) The migration of the solvent from the solid surface to the bulk solvent (Singh et al., 2011). The recovery of bioactive compounds is always poor when using the aforementioned process. The consistency of the compounds extracted is decreased due to the high operating temperatures used during the extraction (Singh and Orsat, 2014).

3.1.2.2. Soxhlet extraction

Another traditional approach used for the recovery of phenolic compounds is soxhlet extraction. A standard soxhlet extraction device that utilizes chemical solvents and heat is shown in Figure 3.6. In the distillation column, the solvent is boiled, where the solvent vapors migrate to the condenser unit, where the solvent condenses into a liquid and falls into the extraction vessel's food supply. Since the solvent extracted is less reactive than the solvent, the solvent is left in the extraction vessel and recycled back into the distillation column (Singh and Orsat, 2014). This cycle is carried out until the end of the extraction. The soxhlet extraction has low operation costs, easy to handle, and does not require filtering after extraction (Wang and Weller, 2006).

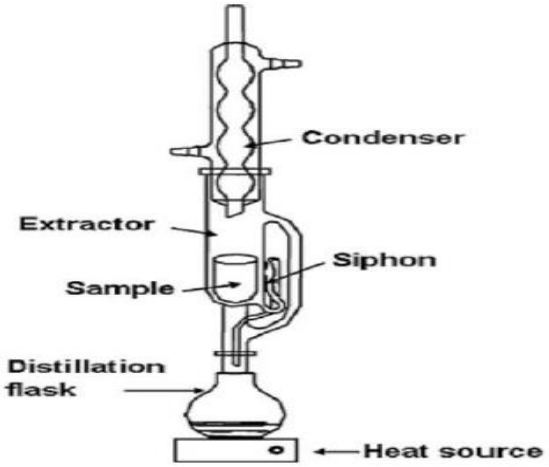


Figure 3.6. Soxhlet extraction apparatus.

3.1.2.3. Ultrasound-assisted extraction (UAE)

The UAE uses sound waves with a frequency greater than 20 kHz and has been used to extract plum flavonoids and polyphenols, ginseng root (Kim et al., 2007), and green walnut husk (Xu et al., 2016). The UAE induces cycles of extension and compression in the extraction medium. The cycles of extension and compression cause the formation of cultivation bubbles in the liquid medium, which expand and collapse during the cycles of expression and compression. Evidently, during a compression period, the cavitation bubble collapses, which disturbs the solid surface, thus increasing the transfer of solvent into the biological matrix and passing solutes to the bulk liquid process (Routray et al., 2013). The UAE is a viable method for the extraction of thermally sensitive compounds because the temperature remains low during this process (Routray and Orsat, 2012)

3.1.2.4. Supercritical fluid extraction (SFE)

SFE is an alternative extraction process that incorporates high temperature and pressure combinations, putting the extraction solvent in a supercritical state, cleaning up temperature and

pressure above their critical values (Camel, 2001). The fluid improves by putting the fluid in a supercritical state, increasing the mass transfer of phenolic compounds into the chosen solvent (Routray and Orsat, 2016). In the supercritical condition, the chosen solvent increases fluidity, which increases the mass transfer of phenolic compounds into the chosen solvent (Routray et al., 2013). In the supercritical condition, the chosen solvent has a lower viscosity than liquids, resulting in a higher diffusion coefficient leading to higher mass transfer for successful extraction. SFE uses non-toxic solvents such as CO_2, which is also cheap, non-flammable, and environment friendly (Routray et al., 2013). The amount of toxins is decreased in the extract by this method. SFE has been used in the extraction of black pepper essential oils, olive leaf phenolic compound (Le Floch et al., 1998) and lycopene from tomato skin (Kassama et al., 2008). SFE has many benefits over traditional methods, such as: a high solving capacity that can be easily controlled by temperature and pressure variations, supercritical fluid diffusivity is better than conventional solvents, high extraction yield, and environmentally friendly as no harmful solvents are used (Singh and Orsat, 2014). However, SFE is expensive to run and difficult to introduce in the industry because of the complex operating requirements.

3.1.2.5. Ultrasonic-assisted extraction

Ultrasonic extraction is the favored technique to isolate bioactive compounds from medicinal plants. Due to its strong cavitation effect, mechanical effect, thermal effect, ultrasonic-assisted extraction is a fast and efficient method. The ultrasonic wave extracts bioactive compounds rapidly and fully, enhancing the biochemical activities of extract (Liu et al., 2018; Ma et al., 2016). Ultrasonic has been used recently by Liu et al. (2018) to extract polyphenolic compounds from okra leaves and rice cultivars, respectively. On the other hand, Özcan and Özkan. (2018) studied total phenol, flavonol, and antioxidant activity in some thyme species growing in Turkey by two extraction methods such as soxhlet and ultrasonic. As a result, the ultrasonic method showed the highest amount of total phenolic content than soxhlet extraction.

As a novel technology, ultrasonic extraction has been proposed by many researchers, such as phenolic compounds from *Justicia spicigera* leaves (Anaya-Esparza et al., 2018), *Oryza sativa* L. (Turrini et al., 2018), *Aver Tunucatum* leaves (Yang et al., 2017).

3.1.3. Analytical methods for non-volatile organic compound

There is no single method established to study the bioactivity of the plant's non-volatile organic compounds. A proper method is essential to primary screen for the presence of the source bioactive compounds. After that, to purify and successively identify the compounds therein. Non-volatile organic compound analysis methods are depending on the target bioactive compound and these may include antioxidant, anticancer, antimicrobial, anti-malarial, seed germination, and many more activates. The analysis technique should be as specific, simple, and rapid as possible (Doughari, 2012). Many problems are created during the phytochemical quality control, standardization, and analyses of herbs and plants due to their complex nature and natural variability of chemical constituents. Therefore, the reproducibility of herbal drug constituent's total formation is very significant, which is alternatively fulfilled by the plant chemo profiling. Non-volatile organic compound standardization means all possible chemical compound information present in the target sample. There are various methods used for the primary phytochemical compound analyses, for example, high-performance thin-layer chromatography (HPTLC), high-performance liquid chromatography (HPLC), ultra-performance liquid chromatography (UPLC), liquid chromatography-nuclear magnetic resonance (LC-NMR), infrared spectroscopy (IR), etc. The spectroscopic and chromatographic techniques are the most authentic tools for phytochemical analysis. Plants contain different classes of bioactive chemical constituents. It is necessary to find out the major non-volatile organic compound classes employing simple chemical tests for different bioactive chemical groups such as phenol, flavonoids, terpenoids, and phytosterols saponin, etc. The following step is to quantify the major classes as they have a bearing on the ability of the plants (Choudhary and Sekhon, 2011).

3.1.4. Optimization of extraction method for total phenol

Using the prediction equations derived by response surface methodology (RSM), the optimum extraction conditions were calculated and used for calculating the expected values for response variables. Verification experiments conducted under the predicted conditions derived from RSM point analysis showed that experimental values were very similar to the predicted values, confirming the predicted model's validity and adequacy (Guido and Moreira, 2017; Topuz et al., 2015). However, the choice of extraction parameters such as temperature, stirring rate, extraction time, particle sample size, pH, and liquid/solid ratio may have a major effect on the recovery of phenols from the medicinal plant (Guido et al., 2017). It is possible to approach the selection of the optimal extraction parameters in one variable in a timely manner, but this technique is extremely time-consuming and potential interactions between variables and parameters are not taken into account at all (Viacaya et al., 2015). In order to optimize the complex process, response surface methodology (RSM) is an important statistical technique. Central composite circumscribed (CCC) design, one form of RSM, is more effective. Optimization experiments are easier to organize and interpret than other designs, and a large number of parameters have been commonly used to optimize (Belwal et al., 2016). To the best of our knowledge, a small number of studies have been performed to optimize the extraction of phenol compounds from medicinal plants and even less to use a green solvent like water. To optimize extraction factors such as temperature, time, and liquid/solid ratio were considered, a systematic approach was used, leading to maximum phenolic compound extraction.

3.1.4. Justification of this study

Scientific research on betel leaf work shows that it has many beneficial biological bioactivities. This extract has a great potential to be used in commercial product production (Wendy Voon et al., 2014; Khan et al., 2013; Catherine et al., 2012; Hoque et al., 2011). Although extensive research on the components has been carried out, few research studies have focused

on maximizing total phenolic compound yield for different purposes (Muruganandam et al., 2017). So it is very important to study maximizing the yield of total phenolic compounds of betel leaves and comparatively to understand their chemical classes, total phenol, total flavonoid, and quantification of major phenolic compounds in the betel leaves varieties. There is no systematic method of extracting and quantifying the major phenolic compound in *piper betel* L. leaves varieties. The first goal was to optimize a high-efficiency method for extracting total phenolic compounds from *Piper betle* L. leaves. Ultrasonic-assisted extraction, a widely used technique for the extraction of bioactive substances from plant materials and food products, was adopted in this research with RSM and CCC design (Wang et al., 2008). Consequently, the second objective was to quantify total phenol, total flavonoid, and major phenolic compound in FBL from Bangladesh.

3.2. Materials and Methods

3.2.1. Samples collection

See details in chapter 1, section 1.2.1

3.2.2. Reagents and chemicals

All pure reagents and chemicals were used in this study, such as chloroform ($CHCl_{3)}$, Sodium hydroxide (NaOH), Sulfuric acid (H_2SO_4), Olive oil, Lead acetate Pb $(C_3H_2O_2)_2$, Acetic anhydride $(CH_3CO)_2O$.

3.2.3. Analytical apparatus of non-volatile organic compounds

The non-volatile organic volatile compound extraction of *Piper betle* L. related apparatus details are shown in Table 3.2.

Table 3.2. Non-volatile organic compound extraction and analysis related apparatus list

S. N.	Apparatus name	Description
i.	Soxhlet	Fisher scientific
ii.	Reflux	Fisher scientific
iii.	Ultrasound	ULTRA-TURRAX, T-25 Basic, IKA Labortechnik
iv.	Soxhlet bath	J-3S3D, Jisico, CO., Ltd
v.	Hot plate & magnetic stirrer	Temperatur Max. 380°C, RPM Max. 1500, Misung Scientific Co. Ltd, Korea
vi.	Ultrasonic bath	Power sonic 420, 40 kHZ, Hwashin technology, Korea,
vii.	Spectrophotometer	Simadzu, UVmini-1240 UV-VIS
viii.	Centrifuge	The Hanil Combi R515, Hanil Scientific Inc, Republic of Korea.
vix.	High-performance liquid chromatography-Diode array detector (HPLC-DAD)	SPD-M20A diode array detector (DAD), LC-20AD pump, CTO-20A column oven, SIL-20 A autosampler, and DGU-20A3R solvent degasser, Simadzu, Japan

3.2.4. Extraction procedure for phytochemical screening

For phytochemical screening, betel leaf sample (Bangla, Sanchi, Misti, Khasia, and BARI Paan 3) were extracted using different extraction techniques:

i) Soxhlet extraction: It was used in different solvents with increasing order of polarity; *n*-hexane, ethyl acetate, and methanol, where solute and solvent ratio was 1:20. The soxhlet extraction bath temperature and time were 70 ºC and 9 hours, respectively. The filtered extracts through Whatman No. 2 (8 µm) and Whatman No. 41 (20 µm) were evaporated to dryness using a rotary evaporator at 40 ºC under reduced pressure for decreasing the volume (1:5) and analysis.

ii) Reflux extraction: 70% methanol was used in reflux extraction where the solute solvent ratio was 1:20. The extraction bath temperature and time were 70 ºC and 2 hours, respectively. The filtered extracts through Whatman No. 2 (8 µm) and Whatman No. 41 (20 µm) were evaporated to dryness using a rotary evaporator at 40 ºC under reduced pressure for decreasing the volume (1:5) and analysis.

iii) Ultrasound extraction: Pure methanol was used in ultrasonic extraction where the solute and solvent ratio was 1:5. The extraction time and centrifuge rotor speed were 10 min and 2000 RPM, respectively. The filtered extracts through Whatman No. 2 (8 µm) and Whatman No. 41 (20 µm) for analysis

3.2.5. Qualitative screening of non-volatile organic compound in FBL

In this study, the crude extracts of *n*-hexane, ethyl acetate, and methanol were qualitatively screened for the detection of various secondary metabolites, for example, phenol, flavonoid, terpene, phytosterol, and saponins, according to the following method Surmaghi et al. (1992) and Sofowara, (1993).

3.2.5.1. Detection of phenolic compound

Lead acetate test: 2 mL of 10% lead acetate solution was added to 3 mL filtrate of the soxhlet crude extracts (1:10 dilution). A white precipitate indicated the presence of phenolic compounds.

3.2.5.2. Detection of flavonoid compound

Sodium hydroxide test: 2 mL of 20% Sodium hydroxide solution was added to 1 mL filtrate of the soxhlet crude extracts (1:10 dilution) and following yellow color shown of flavonoid compounds.

3.2.5.3. Detection of terpene compound

Salkowski test: 2 mL of chloroform was added to 0.5 mL filtrate of the soxhlet crude extracts (1:10 dilution) and then 3 ml of concentrate sulfuric acid was added slowly along the sides of the test tube. A radish interface showed the presence of terpenoids compounds.

3.2.5.4. Detection of phytosterol compound

Liberman-Buchard test: 2 mL of acetic anhydride was added to 0.2 mL filtrate of the soxhlet crude extracts (1:10 dilution) and one drop of concentrate sulfuric acid was added slowly along the sides of the test tube. The array of the color change showed the presence of phytosterols compounds.

3.2.5.5. Detection of saponins

Distilled water test: 5 mL distilled water was added to 0.5 mL filtrate of the soxhlet crude extracts (1:10 dilution) and this solution was vigorously shaken and observed for a stable, persistent froth. The fourth was mixed with 3 to 4 drops of olive oil and shaken vigorously, after which a formation of the emulsion was observed in the test tube presence of saponins compounds.

3.2.6. Optimization of ultrasonic methods for the extraction of total phenolic compound from *Piper betle* L. leaves from Bangladesh

3.2.6.1. Ultrasonic bath and centrifuge condition

0.5 g of the betel leaves powder sample was mixed with a solvent (specific volume and ratio), the solution was extracted by an ultrasonic bath and its working power was fixed at 700 W. The Hanil Combi R515 centrifuge was used to separate sample extract from the solution. The centrifugation condition was fixed in all experiments, such as speed (10000 rpm), temperature (4 ºC), and time (10 min). Finally, the extract was used for the determination of the total phenol content, total flavonoid, and HPLC-DAD analysis. All extraction and test have been operated on a triplicate basis.

3.2.6.2. Extraction solvent selection

In orthogonal design, there were make different solvent ratios by ethanol (70%, 80% and 90%) mixed with acetic acid (2%, 5%, and 10%). Then, 0.5 g of betel leaves powder weighted sample were mixed with 10 mL different ratio of solvents and ultrasonic extraction time and temperature were 30 min and 50 ºC, simultaneously. The extract was prepared for analysis, as mentioned above, after centrifugation (Section 3.2.6.1).

3.2.6.3. Extraction time selection

Over a period of time (20, 40, 60, 80, 100, and 120 min), 0.5 g betel leaf powder weighted sample were ultrasonic extracted with 10 mL ethanol: acetic acid: water (70%: 5%: 25%) (i.e. $V_{ethanol}$: $V_{acetic\ acid}$: V_{water} = 70: 5: 25) solvent at 50 ºC. The extract was prepared for analysis, as described above, after centrifugation (Section 3.2.6.1).

3.2.6.4. Extraction solid/liquid ratio selection

At six different amount of solid/liquid ratio (g/mL) (1:5, 1:10, 1:15, 1:20, 1:2 and 1:30), 0.5 g betel leaf powder weighted sample were ultrasonic extracted with ethanol: acetic acid; water (70%: 5%: 25%) (i.e. $V_{ethanol}$: $V_{acetic\ acid}$: V_{water} = 70: 5: 25) solvent at 50 ºC during 100 min. Extract was prepared for analysis, as described above after centrifugation (Section 3.2.6.1).

3.2.6.5. Extraction temperature selection

At six different temperature (30, 40, 50, 60, 70 and 80 ºC), 0.5 g betel leaf powder weighted sample were ultrasonic extracted with 7.5 mL ethanol: acetic acid: water (70%: 5%: 25%) (i.e. $V_{ethanol}$: $V_{acetic\ acid}$: V_{water} = 70: 5: 25) during 100 min. The extract was prepared for analysis, as described above after centrifugation (Section 3.2.6.1).

3.2.6.6. Extraction of central composite circumscribed (CCC) design

The experiment was planned with three variables (extraction temperature, solid/liquid ratio, and extraction time) and five levels (-1.68, -1, 0, 1, and 1.68) according to the CCC model to select the best variable combination for the determination of highest total phenol compound extraction. The CCC architecture consists of six central points, six axial points, and eight factorial points, creating 20 experimental sets. The independent type of variables (coded and uncoded) are displayed in Table 3.9.

3.2.7. Determination of total phenol (TP)

Folin-Ciocalteu technique described in the study Sazwi et al. (2013) has been adopted to quantify the total phenol amount, with slight changes. In short, 1 mL extract (diluted), 5 mL of Folin-Ciocalteu (10%, v/v), and 4 mL of Na_2CO_3 (7.5% m/v) were mixed together. Then, the mixture was incubated at room temperature for 2 hours, and the absorbance of the mixture was measured at 765 nm using a spectrophotometer (Simadzu, UVmini-1240 UV-VIS). 1 mL blank

absorption was determined at the same condition instead of the extract. A Gallic acid standard was used for establishing a calibration curve (R^2 = 0.9997). The TP amount was reported as a gallic acid equivalent (mg GAE/g DW). The experiments on absorbance were performed in triplicate.

3.2.8. Determination of total flavonoid (TF)

The aluminium chloride colorimetric technique mentioned in Mathur et al. (2107) was adopted in this study with minor modification to quantify the total flavonoid content. In short, 1 ml of extract (dilute) mixed with 3 mL of ethanol (pure), 0.2 mL of potassium acetate (1 M), and 5.6 mL distilled water. Then, the mixture was incubated at room temperature for 30 min followed by the absorbance of the mixture was measured at 415 nm using a spectrophotometer. Out of the extract, 1 mL of blank absorption was determined in the same condition. The quercetin standard was used to create a calibration curve (R^2 = 0.9999). Finally, the outcome was reported as quercetin equivalent (mg QE/g DW). Absorbance tests were performed in triplicate.

3.2.9. HPLC instrument and conditions

A Shimadzu HPLC device consisting of SIL-20A auto sampler, LC-20AD pump, CTO-20A column oven, SPD-M20A diode array detector, and DGU-20A_{3R} solvent degasser was used to qualitative and quantitative analyses. The Capcell core pak C18 column (MG II, 4.6mm I.D. × 250 mm L., 5.0 μm, Osaka Soda, Japan) was used to isolate and separate non-volatile organic compounds. The mobile phase consisted of water (A) and acetonitrile (B) were applied in the gradient elution as follows: linear from 0 to 80 % B (0.00-50.00 min), 80 to 100% B (50.00-50.10 min), 100 to 100% B (50.10-57.00 min), 100 to 10% B (57.00-57.10 min) for qualitative screening of phytochemicals (non-volatile compound) and another linear from 0 to 10 % B (0.00-20.00 min), 10 to 50% B (20.00-30.00 min), 50 to 80% B (30.00-33.00 min), 80 to 90% B (33.00-35.00 min), 90 to 10% B (35.00-40 min) for quantification of major compound. The column was

equilibrated to the starting conditions for 15 min before each run. The injection volume was 10 µL, whereas the column temperature was set at 40 °C and the flow rate was 1.0 mL/min. The chromatographic data were collected and analyzed with Shimadzu LC solution model 1.25 SP4 software. For the determination of phenolic compounds, the wavelength of the HPLC-DAD chromatogram was selected at 280 nm. The amount of the samples were determined from the standard (hydroxychavicol) calibration curve. Each sample analysis was performed in triplicate.

3.2.10. HPLC method validation

The analytical procedure has been validated according to AOAC (The guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals) (Horwitz, 2002). The validation parameters included linearity, sensitivity (LOD and LOQ), specificity, precision, accuracy, and spike recovery.

3.2.10.1. Linearity and sensitivity

The linearity was assessed by linear regression analysis, which was determined by least-squares regression. Ten concentrations (5 to 100 µg/mL) were used for establishing the calibration curves from a standard solution (1000 µg/mL). The standard solution (hydroxychavicol) was diluted with 75% ethanol: 5% acetic acid: 25% water. By plotting each concentration of hydroxychavicol peak area (y axis) versus the concentration (x axis) of each, calibration equations $y = mx + a$, were obtained. A method with the coefficient of determination (R^2) values higher than 0.999 can be considered as linear. For the sensitivity test, the limit of detection (LOD) and limit of quantification (LOQ) were measured from the calibration curves of hydroxychavicol. The LOD and LOQ were calculated using the following equations:

$$LOD = 3.3 \left(\frac{\sigma}{S}\right) \quad \text{……(3.1)}$$

$$LOQ = 10 \left(\frac{\sigma}{S}\right) \quad \text{……...(3.2)}$$

Where, σ = The residual SD of the regression line; S = Slope of the standard curve

3.2.10.2. Specificity and precision

The specificity means the ability of the method to accurately and precisely measure the analyte in the presence of components in the matrix of the sample. It was determined by the chromatogram analysis of the standard and sample solutions. To compare the sample and the reference standard, a diode array detector (DAD) was used in this analysis. The method repeatability (intraday precision) and reproducibility (interday precision) were performed in this analysis. Intraday precision was analyzed from three different concentrations on the same day. Interday precision was analyzed in three replications on three different days. The precession (repeatability and reproducibility) was calculated as percent relative standard deviation (% RSD) for every test by the following equation:

$$\% \text{ RSD} = \left(\frac{SD \times 100}{\bar{X}}\right) \quad \text{.....(3.3)}$$

Where,

SD = Standard deviation of data set

$\bar{X}$ = Mean of data set

3.2.10.3. Accuracy and spike recovery

The accuracy was assessed across the specified range of the analytical procedure by a recovery study. Known standard solutions were used for comparison. Three concentrations (5, 10 and 20 µg/mL) of 100 µL hydroxychavicol were spiked into each 100 µL betel leaf extract to prepare for spike recovery test. The accepted limits for RSD of accuracy is less than 5% with respect to the added amount of sample (Hernández-Fuentes et al., 2010). The percentage of recovery of each compound was analyzed using the validated method. Spike recovery was estimated using the following formulae:

$$\text{Recovery (\%)} = \frac{C_{spiked} - C_{non-spiked}}{C_{added}} \times 100 \quad \text{.....(3.4)}$$

Where,

C_{spiked} = Measured amount after spiking

$C_{non\text{-}spiked}$ = Measured amount before spiking

C_{added} = Actual amount of spike

3.2.11. Statistical analysis

For the investigation, all tests have been showing in triplicate, and the findings were shown in mean ± standard deviation on a sample dry weight basis. The particular factor extraction was investigated by Analysis of Variance (ANOVA), and the total phenol (TP) amount of various extraction treatments were determined by Statistical Package for Social Sciences (SPSS), Software Version 20 (IBM, New York, USA), whereas, $P < 0.05$ was considered as significant. For method optimization to select the best parameter to quantify the highest TP content in betel leaves, the statistic of the central composite circumscribed (CCC) design was done using Design expert 12 (Stat Ease Inc., Minneapolis, USA).

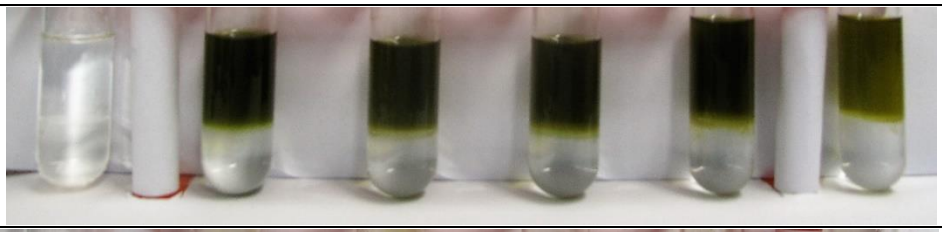
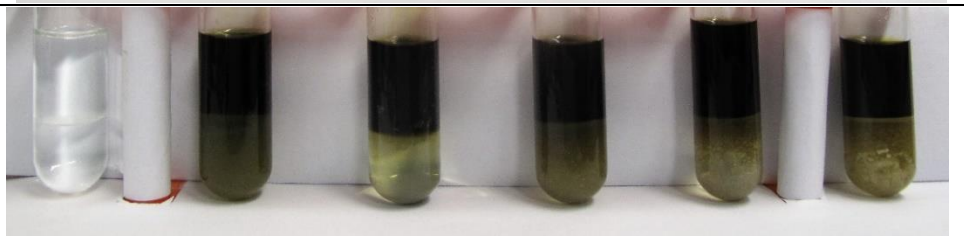
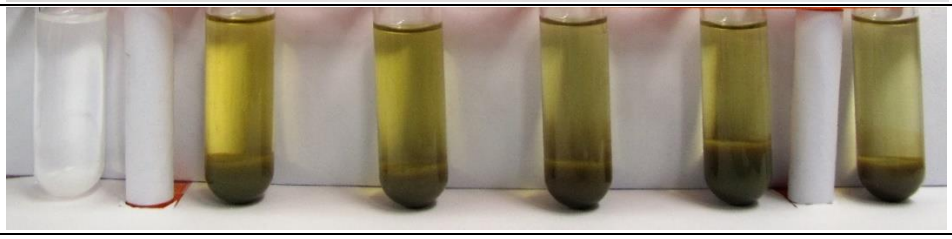
3.3. Results and Discussion

3.3.1. Qualitative screening of phytochemicals (non-volatile organic compound) by chemical test in FBL

The qualitative phytochemicals screening was carried out on FBL from Bangladesh by the chemical test. Secondary metabolites such as phenol, flavonoid, terpene, phytosterols, and saponins are observed in betel leaves which are medicinally important (Table 3.3, 3.4, 3.5, 3.6, and 3.7). Comparative phytochemical screening test observation from the screening taste tube is shown in Table 3.8. Primarily, a positive test was observed (phenol, flavonoid, terpene, phytosterols, and saponins) in FBL.

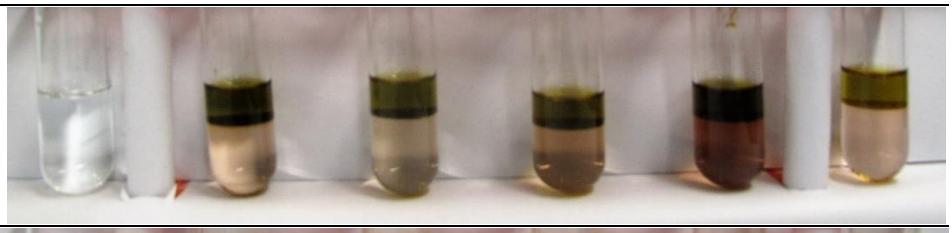
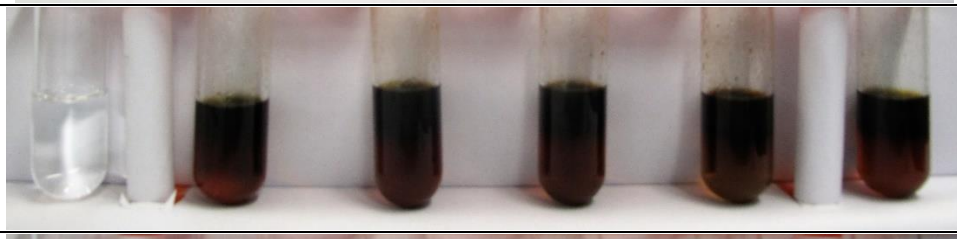
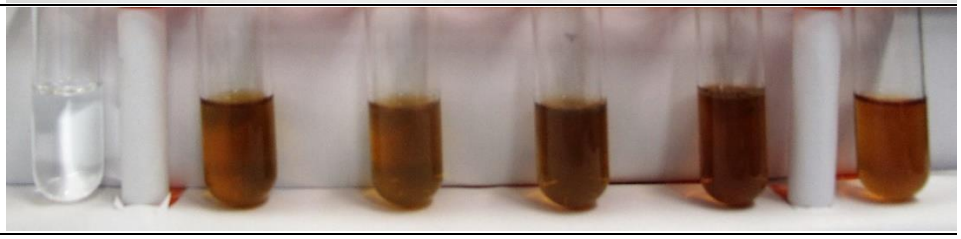
Ethanol and ethyl acetate extract showed the positive test of phenol, flavonoid, and terpene. Simultaneously, hexane extract did not show the positive test of phenol, flavonoid, and terpene. Among the FBL, Khasia leaves showed more phenolic compounds than other varieties. Comparatively, the three solvents, hexane extract, only show the positive test of saponins, oppositely, ethanol and ethyl acetate did not show any positive test of saponins.

Table 3.3. Phenolic compound screening test in FBL

Soxhlet Extraction Solvent Name	Sample name					
	Blank	Bangla	Sanchi	Misti	Khasia	BARI Paan 3
Hexane						
Ethyl acetate						
Ethanol						
Observation from screening test tube						
Hexane	-	-	-	-	-	-
Ethyl acetate	-	+++	+	++	++	++
Ethanol	-	++	++	++	+++	++

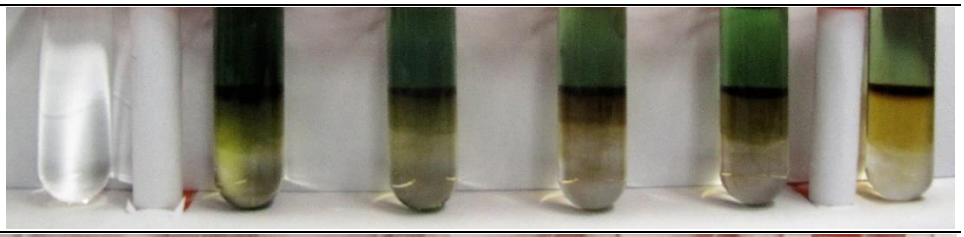
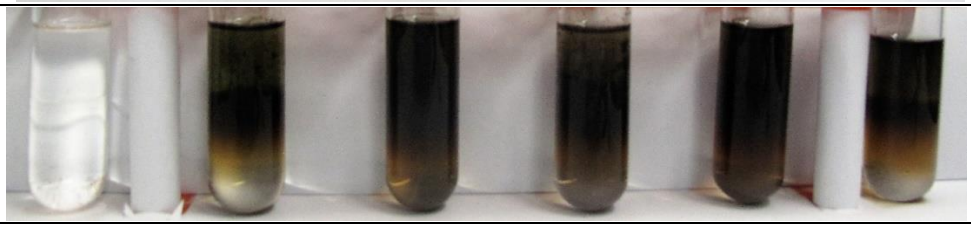
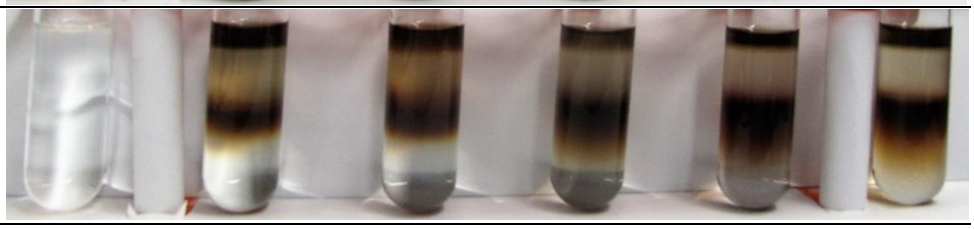
-, did not show positive result; +, show positive result (+, low ; ++, medium; +++, high)

Table 3.4. Flavonoids compound screening test in FBL

Solvent Name	Sample name					
	Blank	Bangla	Sanchi	Misti	Khasia	BARI Paan 3
Hexane						
Ethyl acetate						
Ethanol						
Observation from screening test tube						
Hexane	-	-	-	-	-	-
Ethyl acetate	-	+++	+++	+++	+++	+++
Ethanol	-	+++	+++	+++	+++	+++

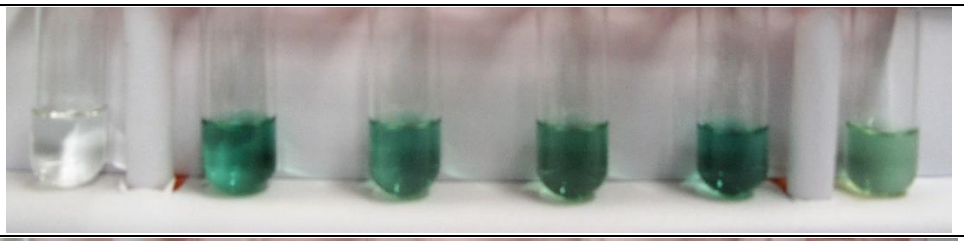
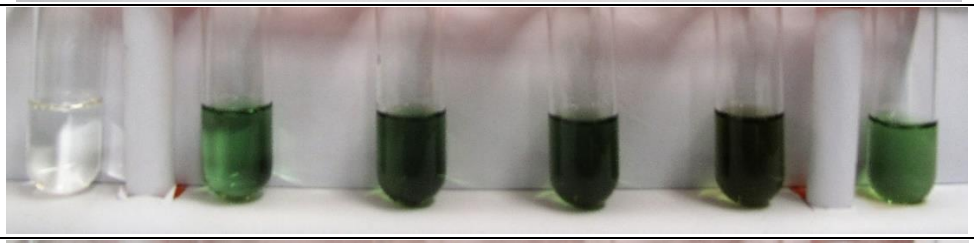
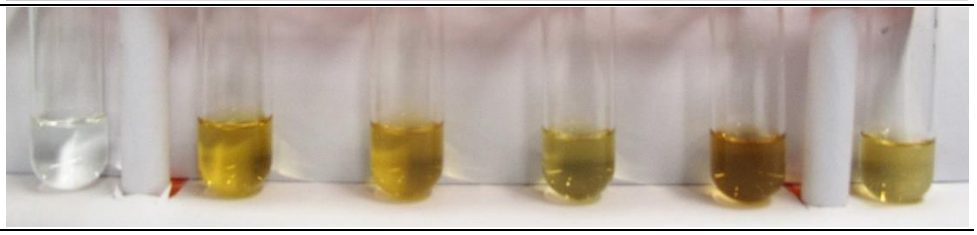
-, did not show positive result; +, show positive result (+, low ; ++, medium; +++, high)

Table 3.5. Terpene compound screening test in FBL

Soxhlet Extraction Solvent Name	Sample name					
	Blank	Bangla	Sanchi	Misti	Khasia	BARI Paan 3
Hexane						
Ethyl acetate						
Ethanol						
Observation from screening test tube						
Hexane	-	+	+	+	+	+
Ethyl acetate	-	++	++	++	++	++
Ethanol	-	++	++	+++	+++	+++

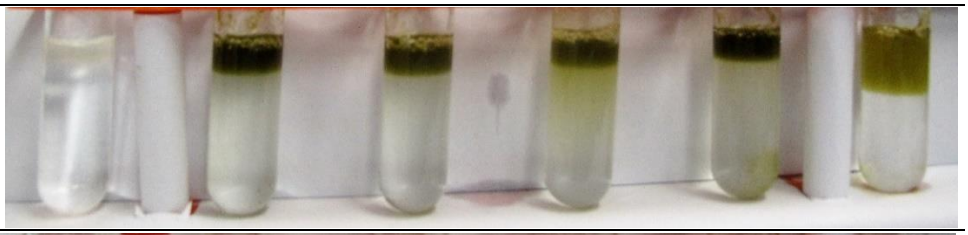
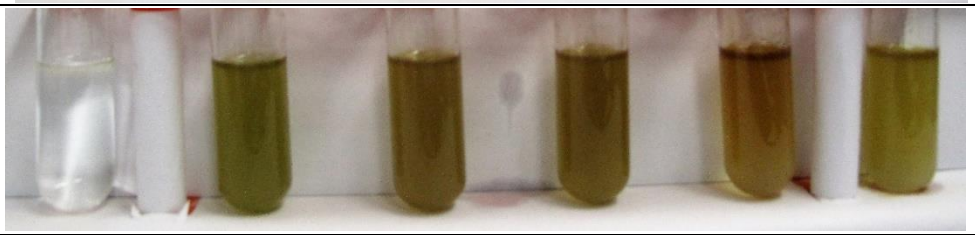
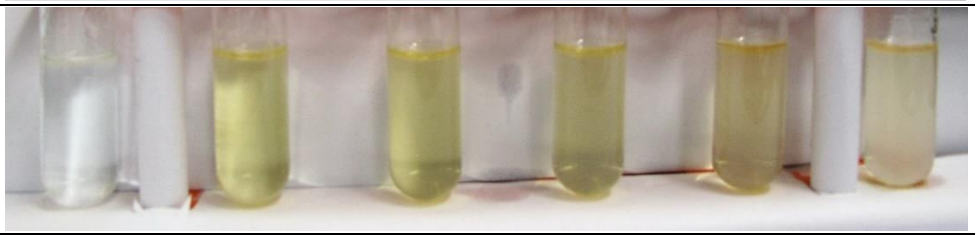
-, did not show positive result; +, show positive result (+, low ; ++, medium; +++, high)

Table 3.6. Phytosterols compound screening test in FBL

Solvent Name	Sample name					
	Blank	Bangla	Sanchi	Misti	Khasia	BARI Paan 3
Hexane						
Ethyl acetate						
Ethanol						
	Observation from screening test tube					
Hexane	-	+++	+++	+++	+++	+++
Ethyl acetate	-	+++	+++	+++	+++	+++
Ethanol	-	-	-	-	-	-

-, did not show positive result; +, show positive result (+, low ; ++, medium; +++, high)

Table 3.7. Saponins compound screening test in FBL

Solvent Name	Sample name					
	Blank	Bangla	Sanchi	Misti	Khasia	BARI Paan 3
Hexane						
Ethyl acetate						
Ethanol						
	Observation from screening test tube					
Hexane	-	++	++	++	++	+++
Ethyl acetate	-	-	-	-	-	-
Ethanol	-	-	-	-	-	-

-, did not show positive result; +, show positive result (+, low ; ++, medium; +++, high)

Table 3.8. Comparative phytochemical screening test results in FBL

Solvent Name	Sample name					
	Blank	Bangla	Sanchi	Misti	Khasia	BARI Paan 3
Observation from screening test tube for phenolic compound						
Hexane	-	-	-	-	-	-
Ethyl acetate	-	+++	+	+++	++	++
Ethanol	-	++	++	++	+++	++
Observation from screening test tube for flavonoid compound						
Hexane	-	-	-	-	-	-
Ethyl acetate	-	+++	+++	+++	+++	+++
Ethanol	-	+++	+++	+++	+++	+++
Observation from screening test tube for terpene compound						
Hexane	-	+	+	+	+	+
Ethyl acetate	-	++	++	++	++	++
Ethanol	-	++	++	+++	+++	+++
Observation from screening test tube for phytosterol compound						
Hexane	-	+++	+++	+++	+++	+++
Ethyl acetate	-	+++	+++	+++	+++	+++
Ethanol	-	-	-	-	-	-
Observation from screening test tube for saponin compound						
Hexane	-	++	++	++	++	+++
Ethyl acetate	-	-	-	-	-	-
Ethanol	-	-	-	-	-	-

-, did not show positive result; +, show positive result (+, low ; ++, medium; +++, high)

3.3.2. Qualitative screening of non-volatile organic compound by HPLC-DAD analysis in FBL

The qualitative phytochemicals screening by HPLC-DAD analysis was carried out in FBL from Bangladesh. The common extraction technique (soxhlet, reflux, and ultrasound) was used for betel leaf extraction, where the solvent was ethanol. HPLC-DAD analysis was performed in the range 190 - 700 nm. The representative fingerprints were showed good chromatographic separation for the most visible peak at 203 nm. The comparatively good chromatographic separation was observed at 230 nm and 280 nm. On the other hand, the minimum separation was shown at 320 nm in all analyzed samples (Figure 3.7, 3.8, and 3.9). At retention time 24.5 min, a board peak was observed in all extract at 203 nm, 230 nm, and 280 nm but 320 nm was not shown any broad peak at retention time 24.5 min. Among the FBL, Khasia leaves show a more visible peak at retention time 24.5 than other varieties.

The phenolic group of secondary metabolites could provide a response to UV range with many absorption intensities for their functional group and respective structural characteristics (Kuppusamy et al., 2018). The selection of appropriate wavelengths is important for each analyte. A single one is insufficient for the simultaneous determination of various compounds in plant extracts. The wavelength range of 230 – 280 nm seems to be used mainly for the simultaneous determination of various phenolic compounds from plant materials (Zang et al., 2013). Many studies have proved that phenolic compound detection's common wavelength was 280 nm (Mizzi et al., 2020; Tsimidou et al., 1992). So, the wavelength 280 nm was selected to determine phenolic compounds in this analysis.

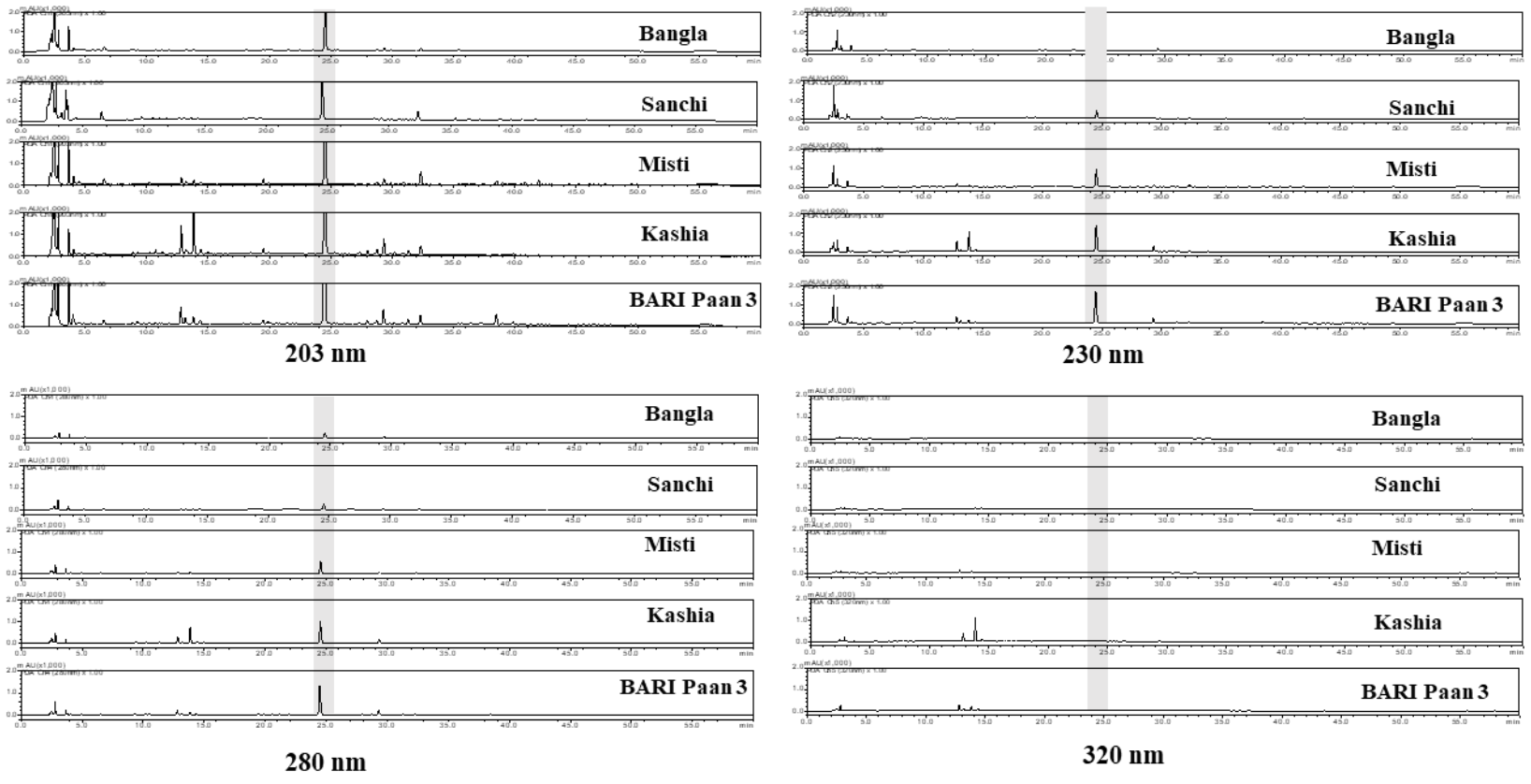


Figure 3.7. HPLC-DAD chromatogram of soxhlet ethanol extraction for non-volatile organic compound screening according to wavelength in FBL from Bangladesh.

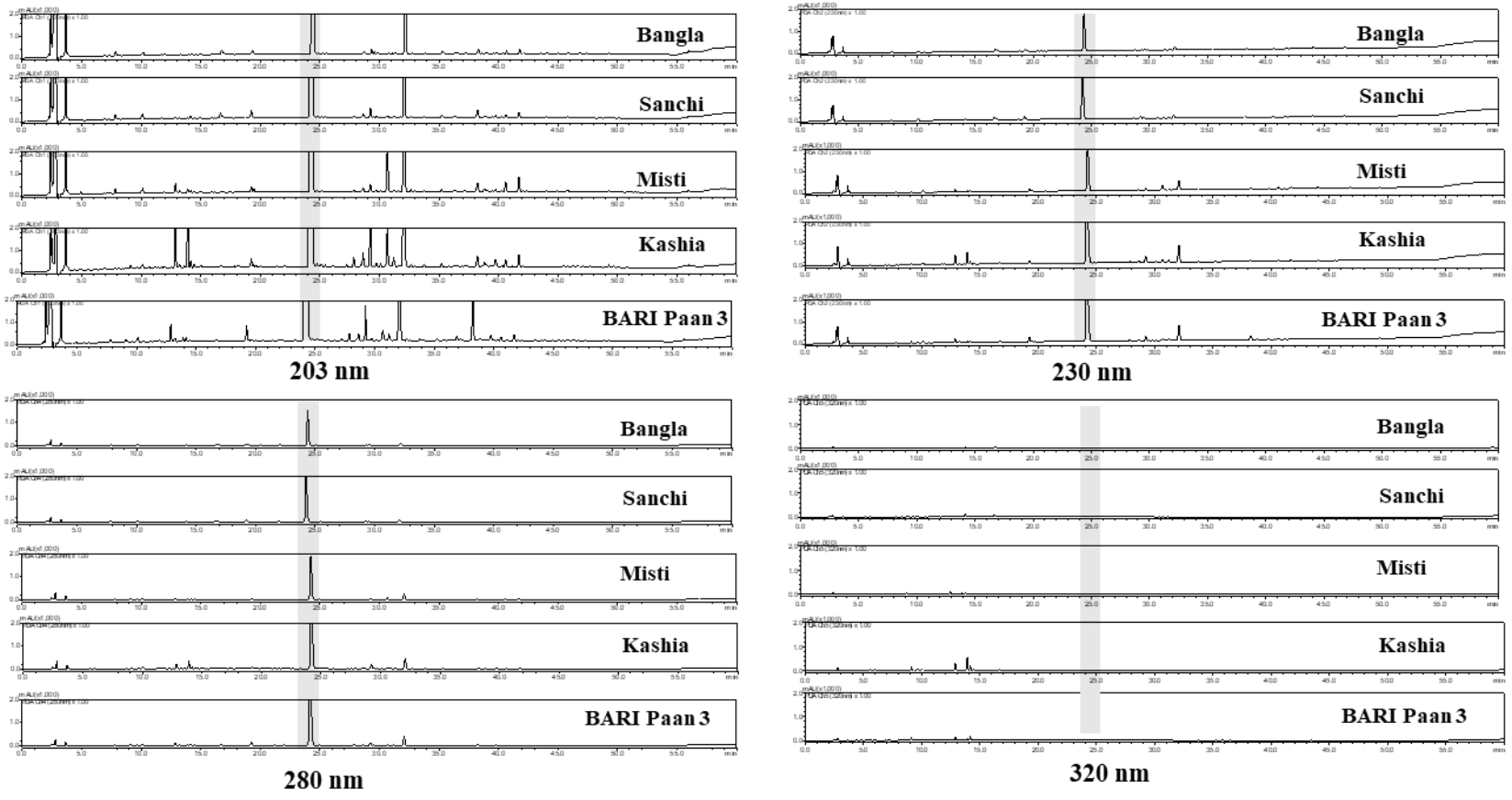


Figure 3.8. HPLC-DAD chromatogram of reflux ethanol extraction for non-volatile organic compound screening according to wavelength in FBL from Bangladesh.

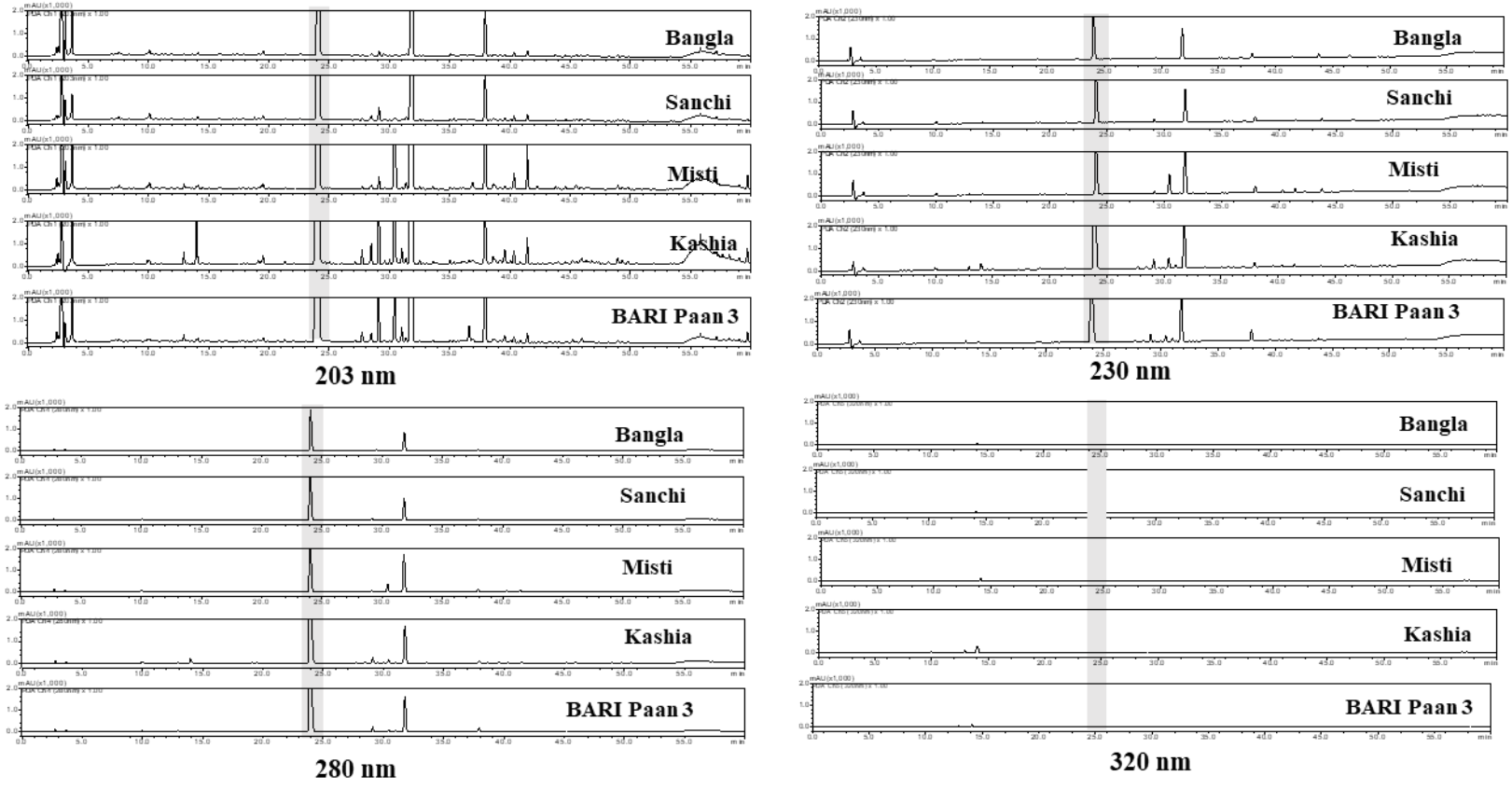


Figure 3.9. HPLC-DAD chromatogram of ultrasound ethanol extraction for non-volatile organic compound screening according to wavelength in FBL from Bangladesh.

3.3.3. Single-factor experimental analysis

3.3.3.1. Influence of solvent ratio to extract total phenol

Generally, different types of solvents (aqueous methanol, ethanol, and acetone) were used to extract the total phenolic compounds from botanical materials, especially from herbs (Wang et al., 2008). Similarly, some acid has also been used to acidify the extraction process for greater efficiency, for example, formic acid, hydrochloric acid, and acetic acid (Tabart et al., 2011). In this analysis, relatively low toxicity solvent ethanol and acetic acid was chosen for the potential future industrial application of phenolic compound extraction. The maximizes of solvent; three separate proportion was selected for the concentration of ethanol (70%, 80%, and 90%) and acetic acid (2%, 5%, and 10%) and their outcomes of total phenolic content presented in Figure 3.10 (a). Meanwhile, other variables (time, 30 min; extraction temperature, 50 °C; and solid/liquid ratio, 1:20 g/mL) were kept constant. According to Figure 3.10 (a), the total phenolic content (TP) of *Piper betle* L. leaves decreased with the increase of ethanol and acetic acid content. The highest TP content (228.55 ± 3.16 mg GAE/g DW) was detected when the extraction solvent ratio was ethanol:acetic acid:water (70%:5%:25%) and other solvent ratio was showed that the total phenol content was lower than this amount. Thus, the solvent ratio ethanol (70%), acetic acid (5%), and water (25 %) were selected in this experiment.

3.3.3.2. Influence of extraction time to extract total phenol

Time is an essential reason for determining the total phenolic compounds extraction. Justifiable extraction time is very important for extracting phenolic compounds, reducing production time, energy consumption, and increasing the yield. On the other hand, too long an extraction period will cause the target compounds to decompose (Naczk et al., 2004). The six times (20 min, 40 min, 60 min, 80 min, 100 min and 120 min) were set to optimize the extraction time, in the meantime, other parameters were kept constant (solid to liquid ratio, 1:20 g/mL; ethanol: acetic acid: water, 70%: 5%: 25%; and extraction temperature, 50 °C). According to

Figure 3.10 (b), the extraction yield of the total phenolic compound in *Piper betle* L. leaves was increased with time and reached the highest (257.52 ± 7.79 mg GAE/g DW) at 100 min and after this point in time, the extraction yield was showed to decreasing trend. Therefore, the extraction time was set at 100 min for the following step.

3.3.3.3. Influence of extraction solid/solvent ratio to extract total phenol

For total phenolic compound extraction, the solid/liquid ratio plays a major role in the mass transfer of extracts between solvent and plant materials (Andres et al., 2020). Selecting a suitable solid/liquid ratio is essential for obtaining the optimal extraction yield of the total phenolic compound. If the solid/liquid ratio is too big, the impurity amount and the operational cost increase. The possible explanation is that the total phenolic compound from betel leaf cannot be completely extracted in a small or high solid/liquid ratio. To optimize the solid/liquid ratio, in this work the solid/liquid ratio was set at 1:5 g/mL, 1:10 g/mL, 1:15 g/mL, 1:20 g/mL, 1:25 g/mL, and 1:30 g/mL. Whereas other parameters were kept constant (extraction time, 100 min; ethanol: acetic acid: water, 70%: 5%: 25%; and extraction temperature, 50 °C). From Figure 3.10 (c), the total phenolic content of betel leaf increased from 234.66 ± 1.48 to 298.78 ± 2.68 mg GAE/g DW when the solid/liquid ratio increased from 1:5 g/mL to1:15 g/mL. Though, on-again increasing the amount of solid/liquid ratio, the total phenolic compound extraction yield was declined. So, the solid/liquid ratio of 1:15 g/mL selected for the next step.

3.3.3.4. Influence of extraction temperature to extract total phenol

The higher extraction temperature, the higher extraction yield, was observed, while molecules travel faster at a higher temperature and result in increased diffusion and permission behaviors (Chimuka et al., 2009). It does negatively affect if the extraction temperature is too high because the heat-sensitive compound will be damaged. As a result, extraction temperatures between 30 °C to 80 °C were studied while other variables were constant (extraction time, 100

min; ethanol: acetic acid: water, 70%:5%:25%; and solid to liquid ratio, 1:15 g/mL). Figure 3.10 (d) showed that the extraction yield from betel leaves for the total phenolic compounds was increased from 30 °C to 70 °C. After that, the further rise in the extraction temperature resulted in total phenolic content steadily decreased. The total phenolic compound maximum concentration was found 271.31 ± 0.34 mg GAE/g DW at 70 °C.

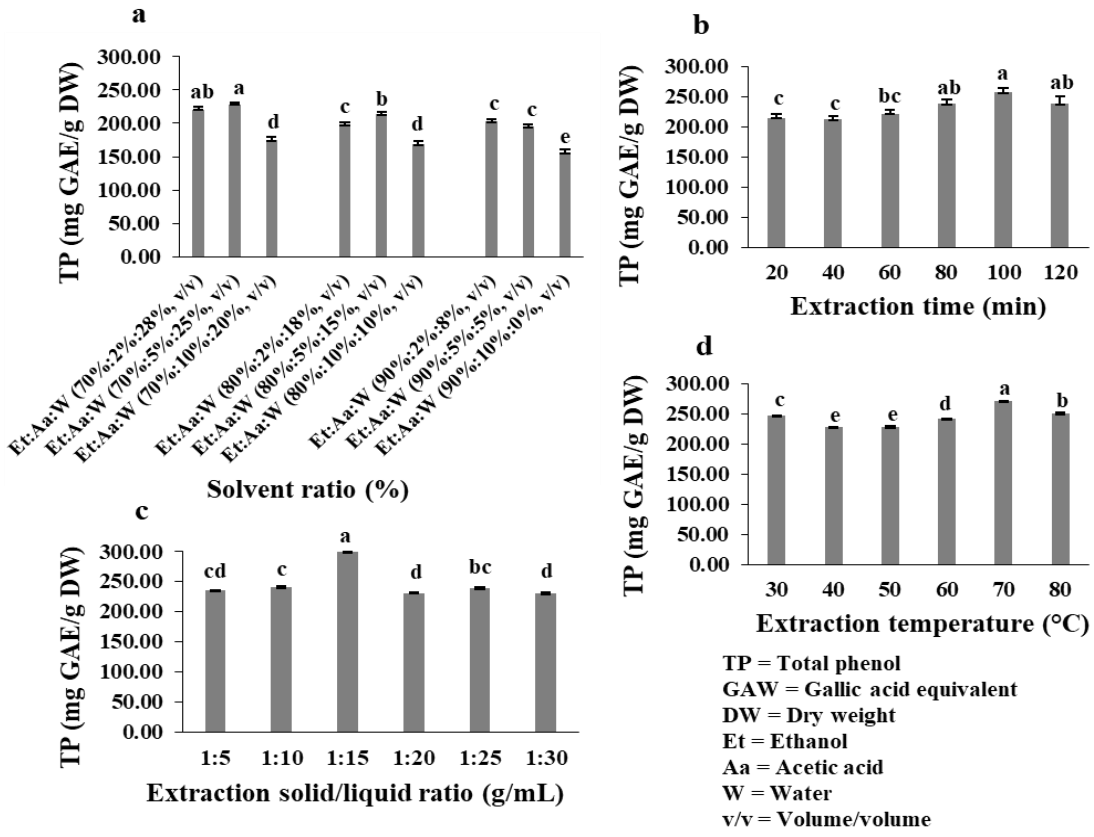


Figure 3.10 (a-d). Effect of single factors; (a) solvent ratio, (b) extraction time, (c) solid/liquid ratio, and (D) temperature on total phenol (TP) content. Means of each two treatments were compared using the least significant difference (LSD) statistic method. Lowercase letters a, b, c, d, and e on the bar diagram were used to mark the significance of difference ($p < 0.05$). Same letters between treatments mean insignificant difference. Different letters between treatments mean significant differences.

3.3.4. Optimization of the variable by central composite circumscribed design

3.3.4.1 Model fitting with statistical analysis

Single-factor studies can only study the effect of change in one factor on the response variable. In this analysis, the central composite circumscribed (CCC) design investigated the impact of the interaction between the three key variables (time, solid/liquid ratio, and temperature) on the extraction yield of the total phenolic compounds. Table 3.9 shows the detailed CCC designs and results. Multiple regression analysis was used to evaluate the correlation of the three variables and the total phenolic compound extraction yield, and a second-order polynomial equation was represented as below:

Total phenolic content (Y) = 286 – 4.90A + 1.84B + 14.83C + 0.9067AB - 4.11AC + 3.00 BC + $3.58A^2$ - $23.81B^2$ – 7.43 C^2…….…… …… (3.5)

Where,

A = Time

B = Solid/liquid ratio

C= Temperature, and

Y = The gross total phenol extraction yield.

In Table 3.10, the parameter was listed from the analysis of variance (ANOVA) table for the design of central composite circumscribed. A model with an *F*-value and *p*-value was 114.71 and <0.0001, which indicated high significance. This model also meant that it was very compatible with the experimental results. The lack of fit of *F*-value and *p*-values were 0.9894 and 0.5390, correspondingly. These values indicate that the lack of fit was not significant compared to the pure error. The coefficient of determination (R^2 = 0.9923) meant that this model could account for 99.23%. The changes in the response value and the fitting precision of this model were satisfactory. The adjustment coefficient (Adj. R^2 = 0.9837) was near the R^2, which means the experiment results fit the predicted results very well. The coefficient of variation (CV %) was 1.26, which meant the model could be replicated (Andres et al., 2020).

In addition, several diagnostic plots also assessed the fitness of the model and internally studentized residuals plots were constructed model's appropriate fit (residual versus run, residual versus block, and residual versus predicted) as presented in figure 3.11 (a-c). It was evident that the values excepted by the model were well corroborated with the experimental values along with the usual percentage probability plot of residuals for the predicted versus actual data to the straight diagonal line and indicated the appropriate relevance with real data Figure 3.11 (d).

3.3.4.2. Optimization of ultrasonic extraction

Figure 3.12 (a-c) shows that three-dimensional response surface plots are drawn by the CCC design and shown to deliver a better graphic representation of the effects of interactions on the response value between the independent variables. The total phenolic compounds extraction yield was obtained for each figure by varying two variables, whereas the other variable is kept constant. Figure 3.12 (a-c) demonstrates the effects of the time, temperature, and solid/liquid ratio pairwise interaction on total phenol extraction's yield. As Figure. 3.12. (a) shows, TP content increased when the solid/liquid rate was increased at the time. Further, comparatively higher TP content was obtained when the solid-liquid ratio was tending 1:15.413 with extraction time managing to 90 min simultaneously. From Figure 3.12. (b), the TP content increased at a specific extraction time after the temperature rise, and the pattern was more evident at the extraction temperature range of 65 to 75 min. Figure 3.12 (c) shows that the closer solid/liquid rate was to 1:15.41 g/mL and the temperature range was 65 to 75 °C for the higher TP amount.

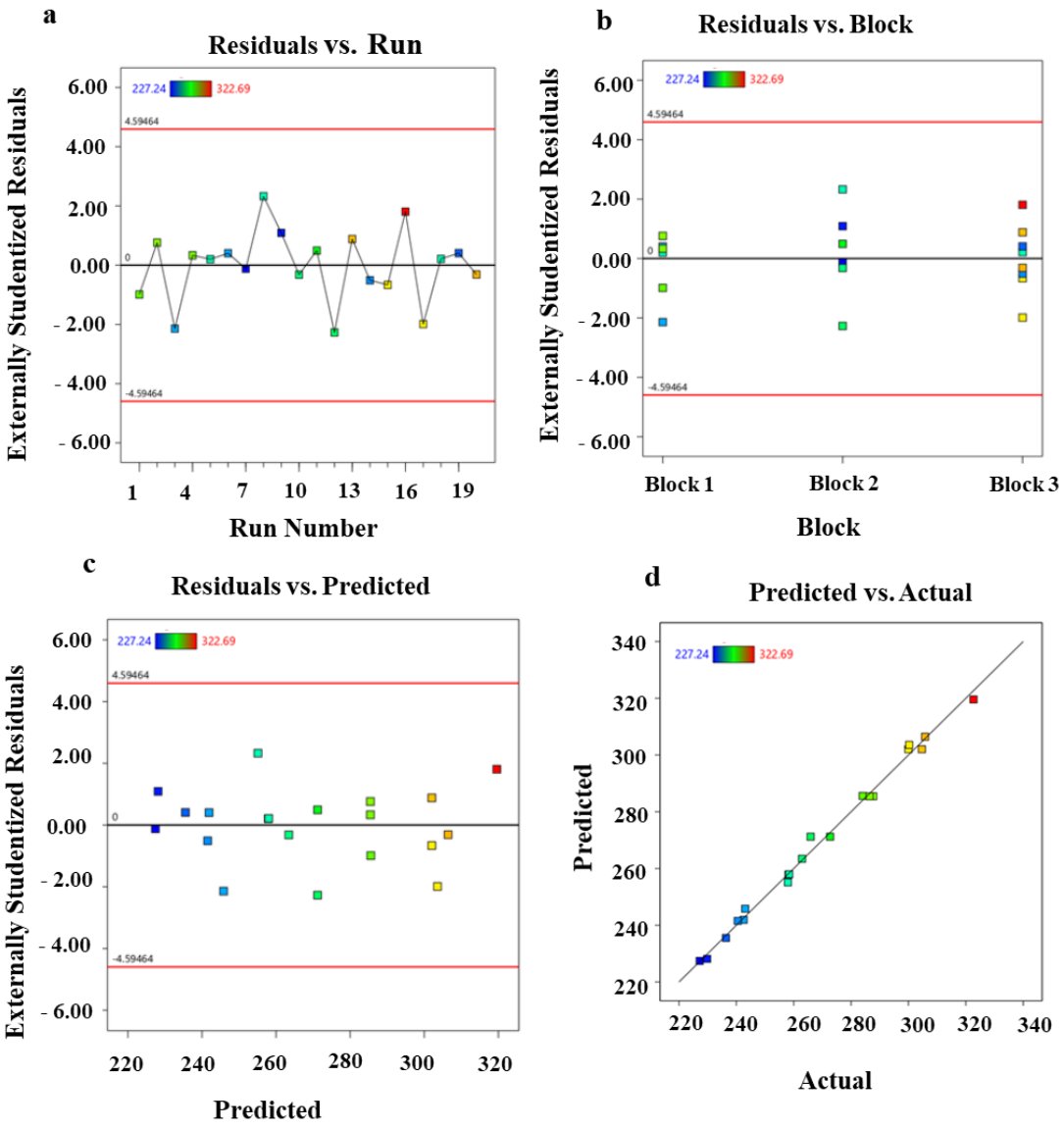


Figure 3. 11. (a-d): Residual plot of CCC design (a) plot of residual versus run, (b) residual versus block, (c) residual versus predicted, and (d) predicted versus actual.

Table 3.9. Central composite circumscribed (CCC) design with actual and predicted response

Run No.	Variable						Total phenol content (mg GAE[1]/g DW[2])	
	Coded			Un-coded			Actual response	Predicted response
	Time (min)	Solid: Liquid (g/mL)	Temp-erature (°C)	Time (min)	Solid: Liquid (g/mL)	Temp-erature (°C)		
1	-1	1	1	90	1:20	75	284.06 ± 19.03[3]	285.58
2	0	0	0	100	1:15	70	287.71 ± 15.1	285.46
3	-1	-1	-1	90	1:10	65	243.1 ± 5.68	245.83
4	0	0	0	100	1:15	70	286.48 ± 13.54	285.46
5	1	-1	1	110	1:10	75	258.2 ± 11.77	257.87
6	1	1	-1	110	1:20	65	242.57 ± 15.05	241.92
7	-1	1	-1	90	1:20	65	227.24 ± 8.37	227.43
8	1	1	1	110	1:20	75	257.98 ± 12.93	255.11
9	1	-1	-1	110	1:10	65	229.82 ± 9.16	228.16
10	-1	-1	1	90	1:10	75	262.92 ± 12.7	263.44
11	0	0	0	100	1:15	70	272.69 ± 11.59	271.2
12	0	0	0	100	1:15	70	265.91 ± 8.28	271.2
13	0	0	0	100	1:15	70	304.68 ± 19.5	302.04
14	0	1.68	0	100	1:23.16	70	240.51 ± 9.51	241.55
15	0	0	0	100	1:15	70	300.00 ± 7.87	302.04
16	-1.68	0	0	83.67	1:15	70	322.69 ± 6.71	319.59
17	1.68	0	0	116.33	1:15	70	300.27 ± 7.08	303.58
18	0	0	-1.68	100	1:15	61.84	258.44 ± 9.91	258.00
19	0	-1.68	0	100	1:6.84	70	236.37 ± 6.53	235.53
20	0	0	1.68	100	1:15	78.16	305.79 ± 9.73	306.44

[1]GAE = Gallic acid equivalent, [2]DW = dry weight of the sample, [3]Mean ± SD (*n* =3)

Table 3. 10. Analysis of variance (ANOVA) for response surface quadratic model for the total phenol extraction

Source	Sum of Squares	df	Coefficient Estimate	*F*-value	Mean Square	*p*-value
Model	11863.98	9	286.23	114.71	1318.22	< 0.0001
Time (A)	320.50	1	- 4.90	27.89	320.50	0.0007
Solid/liquid ratio (B)	45.26	1	1.84	3.94	45.26	0.0825
Temperature (C)	2933.18	1	14.83	255.25	2933.18	< 0.0001
AB	6.58	1	0.9067	0.5723	6.58	0.4710
AC	134.85	1	-4.11	11.74	134.85	0.0090
BC	72.16	1	3.00	6.28	72.16	0.0366
A^2	169.22	1	3.58	14.73	169.22	0.0050
B^2	7487.87	1	-23.81	651.61	7487.87	< 0.0001
C^2	729.50	1	-7.43	63.48	729.50	< 0.0001
Lack of Fit	57.23	5		0.9894	11.45	0.5390
Pure Error	34.71	3			11.57	
Cor Total	15263.03	19				
Standard Deviation	3.39				*R*-Squared	0.9923
Mean	269.37				Adj *R*-Squared	0.9837
C.V. %	1.26				Pre *R*-Squared	0.9317
					Adeq Precision	35.0952

Adj = Adjusted, Pre = Predicted, Adeq = Adequate

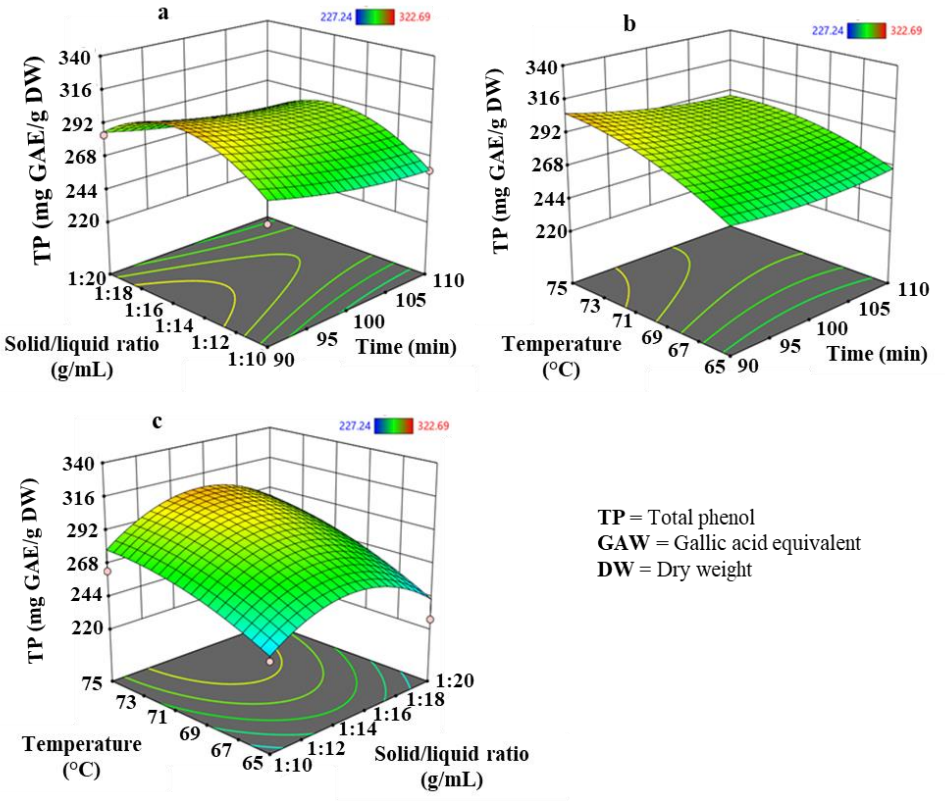


Figure 3.12 (a-c). 3D response surface plots obtained from CCC design on total phenol (TP) content (A) between extraction time and solid/liquid ratio, (B) between extraction time and temperature, and (C) between extraction solid/liquid ratio and temperature.

3.3.4.3. Verification of the predicted model

The total phenolic (TP) optimum ultrasonic extraction condition was obtained from the CCC model is shown in Table 3.11. The extraction temperature, 75 °C; solid/liquid ratio, 1:15.41 g/mL; extraction time, 90 min. The model predicted a maximum TP response was 306.38 mg GAE/g DW under the optimal condition. The CCC model was validated, and TP content was 322.80 ± 2.28 mg GAE/g DW from real experiments (Table 3.11). Some researchers studied total phenolic content in betel leaf by different traditional methods such as cold extraction, shaking incubator, and maceration. The amount was varied from 0.95 to 41.29 mg GAE/g DW (Harini et al., 2018; Saputra et al., 2016; Jaiswal et al., 2014). The total phenol content was determined 2 to 5 times greater than that of previous studies, which means the optimized ultrasonic extraction method is effective for betel leaf phenolic compound extraction.

Table 3.11. Total phenol content from prediction and real experiment under optimal conditions

Optimal condition			Total phenolic content (mg GAE[1]/ g DW[2])	
Time (min)	**Solid/liquid ration (g/mL)**	**Temperature (ºC)**	**Predicted**	**Experimental**
90	15.41	75	306.38	322.80 ± 2.28[3]

[1]GAE = Gallic acid equivalent, [2]DW = dry weight of sample, [3]Mean ± SD (n = 3)

3.3.5. Determination of total phenol and flavonoid in FBL

The FBL have extracted the optimized ultrasonic method conditions. The total phenol and total flavonoid were determined spectrophotometrically with the standard compound of gallic acid and quercetin, respectively. Among the FBL, the TP content was varied from 110.51 ± 1.11 to 322.80 ± 2.28 mg GAE/g DW and the total flavonoid content (TF), was varied 46.79 ± 2.58 to 57.09 ± 6.55 mg QE/g DW (Table 3.12). In this study, the maximum amount TP and TF were recorded 322.80 ± 2.28 mg GAE/g DW and 57.09 ± 6.55 mg QE/g DW, respectively in

Piper betle L. var. Khasia and lowest was 110.51 ± 1.11 mg GAE/g DW and 46.79 ± 2.58 QE/g DW, individually in *Piper betle* L. var. Bangla.

Jaiswal et al. (2014) studied total phenolic compounds with different solvents by shaking for 2 hours about six varieties of betel leaf from India and the TP amount varied from 0.04 to 2.87 mg GAE/g DW basis. On the other hand, Shivashankara et al. (2012) reported total phenols and total flavonoids were 2 to 4 g/100 g and 1.8 to 3.9 g/100 g in three types of betel vine. This amount of TP variation was mainly due to the ultrasonic-assisted method optimization and betel leaf varieties. Another plausible explanation for this was that the amount of phytochemicals depends on many factors, including cultivar variability, environmental conditions, and harvest time (Ghasemzadeh et al., 2018; Tiwari and Cummins, 2013).

Table 3.12. Total phenolic and total flavonoid amount in FBL from Bangladesh

Sample name	Total phenol (mg GAE[1]/g DW[2])	Total flavonoid (mg QE[3] /g DW)
Piper betle L. Bangla	110.51 ± 1.11[4]	51.56 ± 0.47
Piper betle L. Sanchi	175.00 ± 1.83	46.79 ± 2.58
Piper betle L. Misti	191.52 ± 1.80	54.14 ± 3.3
Piper betle L. Khasia	322.80 ± 2.28	57.09 ± 6.55
Piper betle L. BARI paan 3	292.80 ± 2.23	46.41 ± 1.82

[1]GAE = Gallic acid equivalent, [2]DW = Dry weight of sample, [3]QE = Quercetin equivalent, [4]Mean ± standard deviation (n = 3).

3.3.6. Validation of the HPLC-DAD analytical method

3.3.6.1. HPLC-DAD analytical method validation

We optimized the HPLC conditions for the analysis to obtain high separation and resolution of hydroxychavicol. Water and acetonitrile were used as mobile solvents with a gradient elution system to separate and resolution capacity. The detector was set at 280 nm. The chromatogram and spectrum of the hydroxychavicol standard and FBL are shown in Figure 3.14. Good separation could be achieved within 33 min (retention time). The hydroxychavicol standard solution's retention time was around 20.624 ± 0.05 min in FBL from Bangladesh. These results indicate that these HPLC analytical conditions appropriate selectivity and specificity.

3.3.6.2. Linearity and sensitivity

The linearity and sensitivity (LOD and LOQ) results of the method validation for determining hydroxychavicol are shown in Table 3.13. The linearity was determined by analyzing the hydroxychavicol solution in the concentration range of 5 – 100 μg/mL. In Figure 3.13. shows the results for the calibration curve. A linear relationship over the concentration range of 5 – 100 μg/mL was obtained (y = 12180x – 9429.1) with a coefficient of determination was 0.9995. The LOD and LOQ of hydroxychavicol were found to be 3.887 μg/mL and 11.77 μg/mL, respectively.

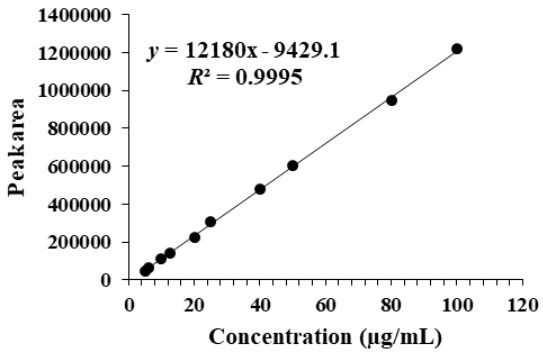


Figure 3.13. Calibration curve of hydroxychavicol by HPLC method.

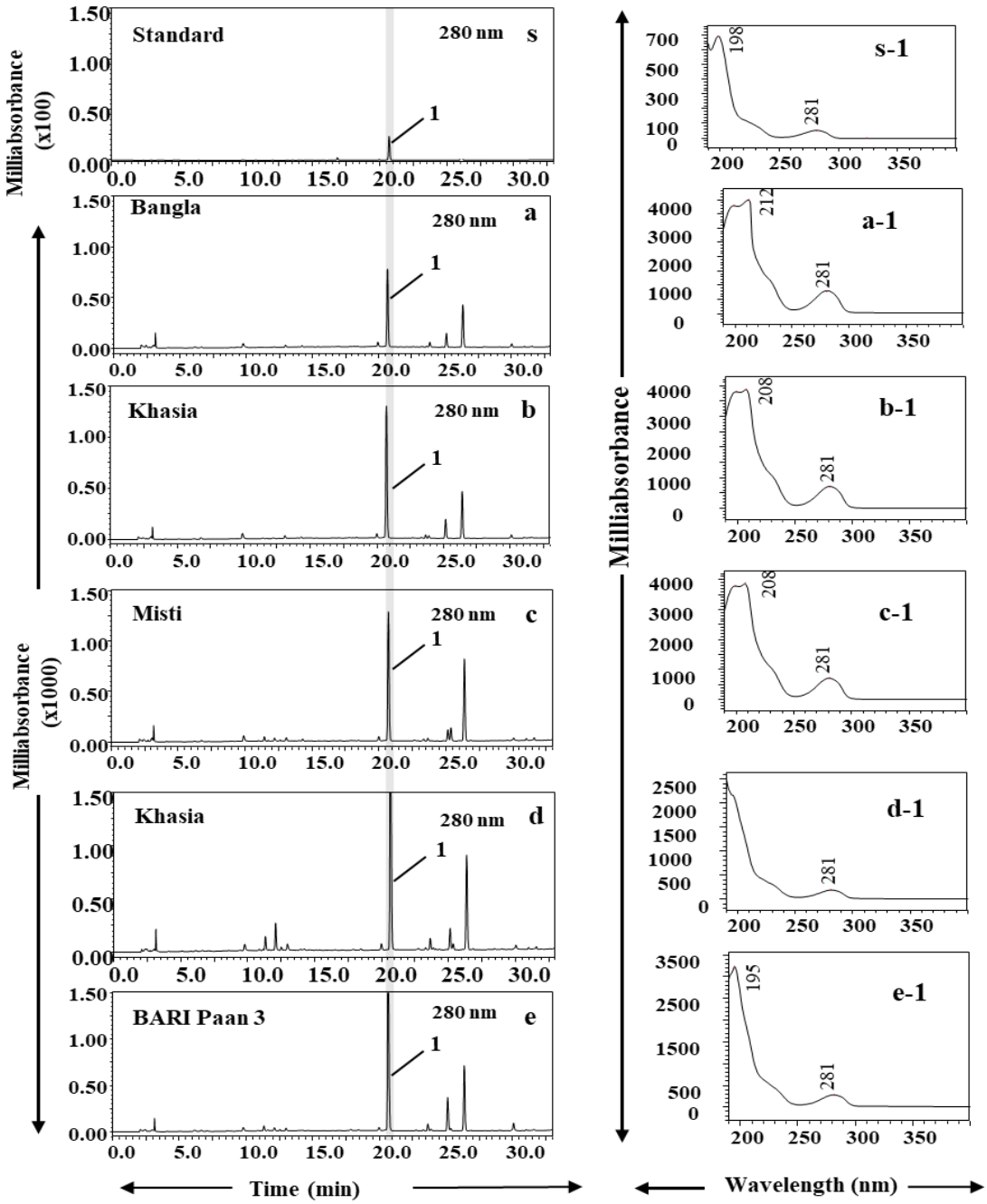


Figure 3.14. (a-f): HPLC-PDA chromatograms and spectrum profile in FBL from Bangladesh. [1 = Hydroxychavicol (RT = 20.56)].

Table 3.13. HPLC-DAD method validation parameters for quantification of hydroxychavicol

Parameters	Hydroxychavicol
Retention time (min; n = 4)	20.63 ±0.05
Regression equation	y = 12180x – 9429.1
Coefficient of determinaion (R^2)	0.9995
Linear range (μg/mL)	5 - 100
LOQ[1] (μg/mL)	11.777
LOD[2] (μg/mL)	3.887

[1]LOQ = limit of quantification, [2]LOD = limit of detection

3.3.6.3. Specificity and precision

In this study, the absence of interferences in the chromatograms of FBL and standard hydroxychavicol at 280 nm confirmed the specificity of the method (data not shown). The method of precision was assessed by measuring relative standard deviation (RSD) intraday (three times a day) and interday (three different days) at three different concentrations (5, 10, and 20 μg/mL) of hydroxychavicol standard, in triplicate. In Table 3.14, the results of intraday and interday precision are presented. Whereas the RSD values are showed less than 2% in intraday and interday precision studies below the limit recommended by the International Conference on Harmonisation (ICH) guidelines (Seo et al., 2016). For hydroxychavicol, the overall recovery range 101.12- 107.33%. These findings showed that the developed method was reproducible with good accuracy.

Table 3.14. Precision and accuracy of hydroxychavicol detection

Analyte concentration µg/mL	Calculated concentration (µg/mL)	RSD[2] (%)	Recovery (%)	Calculate concentration (µg/mL)	RSD[2] (%)	Recovery (%)
	Intraday (n = 3)			Interday (n =3)		
20	21.32 ± 0.08[1]	0.38	106.59	21.46 ± 0.31	1.47	107.33
10	10.75 ± 0.07	0.63	107.53	10.73 ± 0.2	1.89	101.87
5	5.06 ± 0.04	0.88	101.12	5.10 ± 0.07	1.41	101.93

[1]Mean ± standard deviation, [2]RSD = relative standard deviation

3.3.6.4. Accuracy and spike recovery

The accuracy of the validated method was calculated by using a spiking technique. As shown in Table 3.15., the overall recovery of hydroxychavicol ranged between 79.720% to 95.620%, with RSD less than 4%. These values are within the accepted limits, which indicate the applicability of the method for detecting hydroxychavicol in FBL Bangladesh.

Table 3.15. Spike recovery studies of hydroxychavicol in FBL from Bangladesh

Sample name	Amount spiked (µg/mL)	Measured mean concentration (µg/mL)	Mean recovery (%)	RSD[2] (%)
Piper betle L. Bangla	5	4.79 ± 0.14[1]	95.620	2.930
	10	8.27 ± 0.26	82.650	3.146
	20	17.56 ± 0.55	87.810	3.132
Piper betle L. Sanchi	5	4.29 ± 0.09	85.700	2.100
	10	8.86 ± 0.12	88.620	1.354
	20	16.26 ± 0.09	81.320	0.553
Piper betle L. Misti	5	4.62 ± 0.12	91.340	2.582
	10	8.33 ± 0.21	83.620	2.545
	20	16.26 ± 0.09	81.305	0.553
Piper betle L. Khasia	5	4.02 ± 0.14	79.720	3.556

Sample name	Amount spiked (μg/mL)	Measured mean concentration (μg/mL)	Mean recovery (%)	RSD[2] (%)
	10	7.99 ± 0.29	80.610	3.576
	20	18.23 ± 0.36	91.155	1.975
	5	4.77 ± 0.08	95.640	1.757
Piper betle L. BARI paan 3	10	7.98 ± 0.03	79.810	0.376
	20	18.1 ± 0.19	90.475	1.050

[1]Mean ± standard deviation, [2]RSD = relative standard deviation

3.3.7. Determination of hydroxychavicol in FBL by HPLC-DAD

The optimized ultrasonic extract was used to determine major phenolic compounds in FBL, which before used the total phenolic compounds. As shown in Figure 3.14 the hydroxychavicol was detected as a major phenolic compound, which was generally agreed with previous research, which is justified in our study (Syahidah et al., 2017). The content of hydroxychavicol varied according to the varieties of betel leaf. The results indicated the presence of hydroxychavicol 17.44 mg/mL to 38.19 mg/mL in FBL from Bangladesh, which might be the differences in cultivation conditions and variety differentiation. The decreasing order of hydroxychavicol amount was Khasia> BARI Paan 3> Misti> Sanchi> Bangla. Previously, some researchers have reported the presence of hydroxychavicol in betel leaves but not quantify (Pin et al., 2010). This study can quantify the hydroxychavicol amount in betel leaves (Table 3.16).

Table 3.16. Quantification of hydroxychavicol in FBL from Bangladesh

Sample name	Hydroxychavicol (mg/g DW[1])
Piper betle L. var. Bangla	14.19 ± 0.31[2]
Piper betle L. var. Sanchi	20.86 ± 0.24
Piper betle L. var. Misti	17.44 ± 2.88
Piper betle L. var. Khasia	38.19 ± 0.37
Piper betle L. var. BARI paan 3	35.71 ± 0.47

[1]DW = Sample dry weight basis; [2]Mean ± SD

3.4. Conclusion

The non-volatile organic compounds in FBL were successively extracted using a Soxhlet extractor with solvents in the increasing order of polarity for *n*-hexane, ethyl acetate, and ethanol. Preliminary observation confirmed that the betel leaves have secondary metabolites, such as phenol, flavonoid, terpenoids, phytosterols, and saponins. The phenolic compound was higher than other phytochemical classes in FBL from the chemical test. To find out the optimum parameters for the ultrasonic extraction of the total phenolic content from betel leaves, response surface methodology (RSM) with central composite circumscribed (CCC) design was used. The solvent was selected as ethanol: acetic acid: water (70%: 5%: 25%, v/v), and the optimal extraction condition was: time, 90 min; extraction temperature, 75 °C; solid/liquid ratio, 1:15.41. As a result, the total phenolic and flavonoid contents showed high variation ranging from 110.51 to 322.8 mg GAE/g DW and 46.79 to 57.09 mg QE/g DW, respectively in FBL. The maximum total phenol and total flavonoid compound were determined in Khasia betel leaf and the minimum in Bangla betel leaf. HPLC-DAD quantified the major phenolic compound hydroxychavicol 14.19 mg/mL to 38.19 mg/mL in FBL. The highest amount of hydroxychavicol was quantified in Khasia and the lowest was in Bangla betel leaf compared to FBL from Bangladesh. The analytical validation HPLC-DAD method made it possible to detect and quantify the major phenolic marker compound hydroxychavicol in *Piper betle* L. leaves.

CHAPTER IV

Analysis of biochemical activities in five varieties of *Piper betle* L. leaves (FBL) from Bangladesh

4.1. Introduction

Piper betel L. leaves are an economically, medicinally, and traditionally important plant globally. It is a useful asexually propagated crash crop with various varieties (Islam et al., 2020[a]; Patra et al., 2011; WHO, 2004). The betel leaves are mainly used as a mouth freshener with well-known for curing many communicable and non-communicable diseases like cold, cough, bronchial asthma, rheumatism, stomatalgia and used to treat other diseases like bad breath, boils, and abscesses, constipation, swelling of gums, cuts and injuries (Gundala et al., 2014). The betel leaves essential oil possesses anti-fungal, anti-bacterial, and anti-protozoan properties. The aqueous extract of betel vine reduces the adherence of clearly dental plaque bacteria (Punuri et al., 2012). It is also found that betel leaves have significant antiproliferative activity in vitro and in vivo prostate cancer models (Gundala et al., 2014; Bhide et al., 1991). This phenolic compound of betel leaf inhibits prostate cancer through ROS has driven DNA damage and apoptosis (Gundala et al., 2014). HeLa cervical cancer cells were used widely to investigate the anticancer potential of various phytochemical/bioactive molecules. Some of the important constituents of the betel leaf oil are Eugenol, β-caryophyllene, γ-muurolene, valencene, eucalyptol, chavicol, and caryophyllene oxide, and etc (Islam et al., 2020[a]).

4.1.1. Importance of biochemical activity analysis in *Piper betle* L. leaves

Naturally, phytochemicals are present in the plants and playing an important role in protecting themselves and are used in human health to cure different diseases. Phytochemical has antimicrobial activity, which inhibition or killing mechanisms against different pathogenic

microbes and insects. The secretion of these compounds differs from plant to plant, some of which produce more or less. Natural antioxidants can be accepted more readily than synthetic antioxidants. The role of active oxygen and free radicals is more problematic. It is aging and disease processes such as heart disease, inflammation, arthritis, weakening of the immune system. An imbalance between the increased level of reactive oxygen species (ROS) and low activity antioxidant mechanisms describes oxidative stress. Increased oxidative stress can harm the structure of the cell and potentially damage tissues. Antioxidants avoid ROS attacks, thereby helping to prevent illness and health issues. There is a usual trend to look for healthy and efficient natural antioxidants (Lobo et al., 2010). Cytotoxicity studies are a valuable initial step in assessing the possible toxicity of a test substance, including the isolation of plant extracts or biologically active compounds isolated from plants. For the effective production of a pharmaceutical or cosmetic preparation, minimal to no toxicity studies is necessary and cellular toxicity studies play a crucial role in this respect (McGaw et al., 2014). The antioxidant, antimicrobial, and cytotoxicity activity in FBL from Bangladesh is unknown. This chapter focused on the comparative determination of antioxidant, antimicrobial, and cytotoxicity properties in FBL from Bangladesh and their major bioactive phenolic compounds hydroxychavicol and eugenol.

4.1.1.1. In vitro antioxidant activity

Natural antioxidants, for example, total phenolic, total flavonoid, anthocyanins, tannins, and etc have potent antioxidant activity and they eliminate reactive oxygen species (ROS) or free radicals like hydroxyl radical ($OH^{\bullet}$), superoxide ion ($O_2^{-\bullet}$), nitric oxide radicals from our body. The amount of free radicals and antioxidants in the body is usually balanced but can be imbalanced in response to environmental factors, for example, smoke, pollutants, and solvent (Salehi et al., 2020; Ullah et al., 2019**)**. These free radicals may cause numerous diseases in this state, such as cardiovascular diseases, neurodegenerative disorders, a decline

of the immune system, diabetes mellitus, and cancer, and shorten the shelf life of food (Maddu, 2019). In the prevention of these diseases, antioxidants are intimately involved. Synthetic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are widely used as food preservatives. Whereas BHT and BHA are suspected of having abnormal effects on enzyme systems (Ousji and Sleno, 2020). Many studies have shown that betel leaves were in vitro and in vivo systems able to have counteracted oxidative stress (Paranagama et al., 2020; Aara et al., 2020). The antioxidant activities of all the sample crude extracts were evaluated by free radical scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis-3-et hylbenzthiazoline-6-sulphonic acid (ABTS). Nenadis et al. (2004) estimated that the antioxidant activities of the phenolic compound are largely depicted by their molecular structures and their assays.

4.1.1.1.1. DPPH radical scavenging assay

The use of the stable free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH) is based on this procedure. The anti-radical activity of distilled phenolic compounds and natural plant extracts is also commonly used to determine them. A stable free radical with maximum absorption at 515 nm in the DPPH molecule. The spare electron's delocalization over the entire molecule is stable, which prevents dimerization and gives deep violet colour to DPPH. If a DPPH solution is combined with a hydrogen donor substance (antioxidant), during this time, DPPH is reduced and the loses of violet colour (Floegel et al., 2011). The reaction of the mechanism is shown below:

$$DPPH^{\bullet} + AH \rightarrow DPPH + A^{\bullet}$$

$$DPPH^{\bullet} + R^{\bullet} \rightarrow DPPH\text{-}R$$

When a radical is quenched by an antioxidant and the absorbance at 515 nm, the DPPH solution's colour changes from deep purple to a light yellow. The reduced absorbance indicates

the antioxidant activity of the sample tested.

4.1.1.1.2. ABTS radical scavenging assay

The other free radical approach is the use of the chemical procedure is 2,2´-azino-bis-3-et hylbenzthiazoline-6-sulphonic acid (ABTS) which is typically used for the screening of complex phenolic antioxidant mixtures for example, plants, food, and etc. In the ABTS assay test, the antioxidant capacity to scavenge the aqueous phase of the blue-green ABTS radical (absorbed at 734 nm) was produced by reacting with the ABTS salt strong oxidizing agent (potassium persulfate, $K_2S_2O_8$). With the loss of its longwave absorption by an antioxidant, this blue radical decreases. The antioxidant potential is measured as the antioxidant capacity of ascorbic acid. ABTS generates the free radical and the mechanism of DPPH and ABTS is similar. This method is also easy and can be used in both aqueous and organic medium over a wide range of P^H (Ácsová et al., 2019).

4.1.2. Antibacterial activities

Antibacterial resistance to antibiotics is a big problem worldwide. According to the United States, antibacterial resistance is projected to cause more than 700 thousand deaths annually and this Figure is predicted to rise to 10 million worldwide by 2050 (Breijyeh et al., 2020). New antibacterial drug production is an urgent need and it is considered a strong priority. Recent developments are now focusing on natural products and discover new therapeutics for pathogens with flight resistance. The use of phytochemicals and their plant extract is used as antimicrobial agents have been well known and it was studied worldwide for human medicine (Górniak et al., 2019). Plant extracts may function either as bacteriostatic agents (preventing bacterial growth) or as bacterial agents (killing bacteria) and provide useful natural antimicrobial sources. In contrast to synthetic pharmaceuticals, the find for new antimicrobial agents from natural origin is ongoing (Jackson et al., 2018). Antimicrobial assays are evaluated using the

following methods, measuring the bacterial growth inhibition zone around the filter paper disk impregnated with the test samples. The antibacterial activities in this study were assessed by the methods of disk diffusion (DD).

4.1.3. Cytotoxicity activities

In vitro studies, cytotoxicity is one of the essential indicators for biological sample evaluation. Cultured cells are commonly used for chemical cytotoxicity studies and drug screening (Aslantürk et al., 2017). In recent years, the application of these assays has been of growing. Cell viability and cytotoxicity assays are based on several cell functions. There are many methods to determine cytotoxicity assays, such as i) colorimetric assays ii) dye exclusion assays iii) luminometric assays and iv) fluorometric assays. It is important to choose the right technique from these methods to obtain accurate and reliable results. In this analysis, a colorimetric assay was selected to analyze cytotoxicity activities in FBL with their major phenolic compound hydroxychavicol and eugenol. The colorimetric assay is superior to other methods because it is simple to use, safe, and highly reproducible. It is commonly used to assess both cell viability and cytotoxicity tests (Do et al., 2014). The concept of colorimetric assays determines a biochemical marker to evaluate the cells' metabolic function. In response to the cell viability, reagents are used in colorimetric assays to produce a colour that allows the colorimetric measurement of cell viability via a spectrophotometer.

4.1.4. Justification of this study

Betel leaves phytochemicals is known for different medicinal values, and it is used globally in folk medicines but their phytochemical types and concentration depend on their varieties and growing conditions (Islam et al., 2020[a]). Betel leaves are used in Bangladesh, India, Pakistan, Nepal, and etc countries by local Hakims for treatment and control of various diseases. In the previous chapter, phytochemical (volatile and non-volatile organic compounds) analysis

results showed that many potentially bioactive compounds and their amounts are different from one variety to another. However, the comparative biochemical studies in FBL from Bangladesh and their major phenolic compounds hydroxychavicol and eugenol have not been studied before. Therefore, this research was planned to analyze biochemical (antioxidant, anti-microbial, and cytotoxicity) properties in FBL from Bangladesh with their major phenolic compound (hydroxychavicol and eugenol). These findings can help to allow more precious and effective use of the subject betel leaf varieties based on biological properties.

4.2. Materials and Methods

4.2.1. Sample collection

See details in chapter 1, section 1.2.1

4.2.2. Reagents and chemicals

All pure regents and chemical were used in this study, such as ascorbic acid; 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2´-azono-bis(3-ethylbenzo-thiazoline-6-sulfonic acid diammonium salt) (ABTS), potassium persulfate; mueller hinton broth (MHB), dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), sodium dodecyl sulfate (SDS), dimethyl formamide (DMF), thiazolyl blue formazan (MTT). The pure compound hydroxychavicol and eugenol have been collected from Chromadex (Los Angeles, USA) and Sigma-Aldrich (Saint Louis, USA), respectively.

4.2.3. Analytical apparatus of biochemical properties test

The biochemical properties of *Piper betle* L. related apparatus details are shown in Table 4.1.

Table 4.1. Biochemical properties related apparatus list

S. N.	Apparatus name	Description
i	Ultrasonic bath	Power sonic 420, 40 kHZ, Hwashin technology, Korea,
ii	Spectrophotometer	Simadzu, UVmini-1240 UV-VIS
iii	96 microplate absorbance reader	Spectra Max 190, 96 well microplate reader (Molecular Devices, CA, USA
iv	Hematocytometer	Marienfeld Superior, Germany
v	Microscope	Olympus M021, Japan
vi	Cell culture room	Vision Scientific, Korea

4.2.4. Extraction of sample

4.2.4.1. Antioxidant and cytotoxicity activity

See details in chapter 3, section 3.2.6

4.2.4.2. Antimicrobial activity

The optimized ultrasonic betel leaf extract was a little modified for the antimicrobial activity test. In addition, the optimized ultrasonic betel leaf extract was evaporated by a rotary evaporator at 40 ºC. Then, pure DMSO was used to dissolve (Solute: Solvent, 1:1) crude extract. After that, 5% DMSO was used to make the final solution 100 mg/mL for the antimicrobial test.

4.2.5. Determination of antioxidant activities

4.2.5.1. DPPH assay procedure

The DPPH testing method was adopted from Do et al. (2014), with minor modifications, to determine the radical scavenging capacity. In short, 1 mL of appropriately diluted extract and 2 ml of DPPH solution (0.004%, w/v) were mixed and the sample mixture was incubated for 30 min at room temperature. Then the sample absorbance was measured at 517 nm using a Simadzu UVmini-1240 UV-VIS spectrophotometer. The percent of inhibition was calculated using the following formula:

$$\% \text{ inhibition} = \frac{(A_{Control} - A_{Sample}) \times 100}{A_{Control}} \quad \text{.....(4.1)}$$

Where,

$A_{Control}$ = absorbance of DPPH solution without extract.

A_{Sample} = absorbance of sample with DPPH solution.

The half-maximal inhibitory concentration (IC_{50}) was reported by plotting the inhibition (%) against the extract concentrations. Absorbance tests were performed in triplicate.

4.2.5.2. ABTS assay procedure

The ABTS radical scavenging activity was calculated by measuring the ABTS radical cation disappearance, following the process was used by Jan et al. (2013). ABTS (7 mM) and potassium persulfate (2.4 mM) were combined in equal quantities to make the stock solution and put at room temperature in the dark for 12-16 hours. After that, $ABTS^+$ solution (1mL) and 60% methanol (50 mL) solution were added for dilution and absorbance was 0.708 ± 0.001 units at wavelength 734 nm using the Simadzu UVmini-1240 UV-VIS spectrophotometer. The decline in absorbance was taken after 1 minute up to 6 minutes. At that time, the final absorbance was noted. The percentage of inhibition was calculated using the following formula:

$$\% \text{ inhibition} = \frac{(A_{Control} - A_{Sample}) \times 100}{A_{Control}} \quad \text{.....(4.2)}$$

Where,

$A_{Control}$ = absorbance of ABTS solution without extract.

A_{Sample} = absorbance of sample with ABTS solution.

The half-maximal inhibitory concentration (IC_{50}) was reported by plotting the inhibition (%) against the extract concentrations. Absorbance tests were performed in triplicate.

4.2.6. Determination of antibacterial activities

4.2.6.1. Microorganisms for antibacterial activity

The test microorganisms for antibacterial activates include five Gram-negative bacteria; *Escherichia coli* KCTC 1923 (*E. coli)*, *Pseudomonas aeruginosa* KCTC 1637 (*P. aeruginosa*), *Salmonella typhimurium* KCTC 1925 (*S. typhimurium*), *Alcaligenes faecalis* ATCC 1004 (*A. faecalis*), *Extended-spectrum beta-lactamase* W1, (ESBL) and Six Gram-positive bacteria; *Staphylococcus aureus* KCTC 1928 (*S. aureus*), *Methicillin-resistant Staphylococcus aureus* B15 (MRSA), *Mycobacterium smegmatis* ATCC 9341 (*M. smegmatis*), *Micrococcus luteus* ATCC 9341 (*M. luteus*), *Bacillus subtilis* ATCC 6633 (*B. subtilis*), *Vancomycin-resistant Staphylococcus aureus* (VRSA*)* collection from the department of pharmacy, Chosun University, Gwangju, Republic of Korea.

4.2.6.2. Preparation of bacterial culture

IN Mueller Hinton Broth (MHB) an inoculum of bacteria from the bacteria culture was prepared and incubated at 37 ºC for 3 hours. The cultures of bacteria were standardized to the 0.5 McFarland standard turbidity, which was used for disc diffusion assays.

4.2.6.3. Determination of zone of inhibition

The antibacterial activities of the betel leaves extract were evaluated using Kirby-Bauer disc diffusion method (Hudzicki, 2009). With the uniform bacteria suspension, the

surface of MHB prepared in petri dishes was inoculated. The filter paper discs were 8 mm, which are impregnated with 40 µL of 100 mg/mL (dry weight basis of betel leaf sample) and the pure compound of hydroxychavicol, eugenol, and ciprofloxacin concentration was 500 µg/mL. Ciprofloxacin was used as a positive control. The dried filter discs were then placed on the growth agar's surface and incubated at 37 ºC for 24 h. The inhibition zone was then evaluated by measuring the diameter in mm around the filter discs of the clear zone formed.

4.2.7. Determination of cytotoxicity activities

4.2.7.1. Cytotoxicity screening

Human epithelial cells (HeLa), human neuroblastoma clonal cells (SH-SY5Y), mesenchymal stem cells (MSCs) were obtained from American Type Culture Collection (ATCC). The cells were removed from the incubator and microscopic observation of the cells was conducted. Cells were maintained Dulbecco's modified Eagles medium (DMEM) with 10 percent fetal bovine serum (FBS), 100 IU/mL penicillin and 10 µg/mL streptomycin in a humidified atmosphere of 5 percent CO_2 and 95% air at 37 ºC. Cells were shaken gently on the platform in order to determine viability and 70 percent ethanol was thoroughly sprayed on the dishes and flask containing DMEM until inserted into the fume hood. Cells were rinsed 3 times to remove all debris with 2 mL of phosphate buffer saline (PBS) (without salt) and cells leave attached to the surface of the dish. Followed by, 2.0 mL of trypsin was added to the cell culture dish to detach the cells and incubated for 2 minutes. After detaching the cells from the plate, 4 mL DMEM media was added to the cell culture plate to neutralize tripsine. Finally, it was collected in a 15 mL tube and centrifuged at 800 rpm for 5 minutes. Then, media was discarded using a suction pump to get the cell pellet. Again, 4 mL PBS was added to the cell pellet and centrifuged again to get a fresh cell pellet.

4.2.7.2. Cell count

50 µL sample contain cells were pipetted into Eppendorf tubes from 1 mL of DMEM supplement solution containing FBS and diluted it 32 times. Therefore, 10 µL solution was pipetted into two sides of the slide of the hematocytometer and cells were counted under the light microscope. 96 well plates (15000 cells per well) were filled and incubated for 24 hours with 100 µL of supplemented media. Different concentrations of plant extracts (0.08, 0.10, 0.13, 0.17, 0.25, and 0.50 mg/mL) were given in 96 well plates.

4.2.7.3. MTT assay

a phosphate-buffered saline (PBS) solution of 20 µL and 5 mg/mL thiazolyl blue tetrazolium bromide (MTT) was applied to each well and incubated for 2 h. Then, 100 µL of solubilization buffer (20% SDS) was applied to each well and incubated for additional 22 hours. After 24 h at 570 nm, the absorbance was recorded by a Spectra Max 190, 96 well microplate reader (Molecular Devices, CA, USA). The cell viability was measured using the following formula:

$$\text{Cell viability (\%)} = \frac{A_{sample} \times 100}{A_{Control}} \quad \text{.....(4.3)}$$

Where,

$A_{Control}$ = absorbance of the untreated cells.

A_{Sample} = absorbance of the cells treated with the sample.

A control group (medium without samples, 100% viability) and a blank group (without cells, 0 % viability) were also included (Wogulis et al., 2005). All the experiments were done in triplicates.

4.2.8. Statistical analysis

All tests have been shown in triplicate for the investigation, and the findings were shown in mean ± standard deviation on a sample dry weight basis. Analysis of Variance (ANOVA) investigated the particular factor extraction, and the antioxidant and cytotoxicity activity of various extraction treatments were determined by Statistical Package for Social Sciences (SPSS), Software Version 20 (IBM, New York, USA), whereas, $P < 0.05$ was considered as significant.

4.3. Results and discussion

4.3.1. Antioxidant activity in FBL

Recently, a wide range of spectrophotometric assay has been adopted to measure the antioxidant capacity of plant, foods, and pure compounds, the most popular being DPPH (1,1-Diphenyl-2-picrylhydrazyl radical) and ABTS [2,2´-azono-bis(3-ethylbenzo-thiazoline-6-sulfonic acid diammonium salt)] assy, among others such as ferric reducing ability of plasma (FRAP) and oxygen radical absorbance capacity (ORAC) assay (Floegel et al., 2011). Both DPPH and ABTS are stable free radicals dissolving in methanol or ethanol, with typical absorptions at 517 nm and 734 nm, respectively, in their colors. The DPPH and ABTS assay solution colors become brighter as an antioxidant scavenges the free radical by giving hydrogen. As shown in Figure 4. 1, the percent of DPPH and ABTS inhibition values were dose-dependent and increased in the test range. The antioxidant activity of DPPH IC_{50} and ABTS IC_{50} assay was ranged from 0.17 ± 0.01 to 0.43 ± 0.01 µg/mL and 0.04 ± 0.00 to 0.11 ± 0.01 µg/mL, respectively in FBL extract, which compared the ascorbic acid DPPH IC_{50} and ABTS IC_{50} values were 9.12 µg/mL and 5.14, individually (Table 4. 2). The antioxidant activity decreasing trend in FBL was Khasia > BARI Paan 3 > Misti > Sanchi > Bangla. On the other hand, the DPPH IC_{50} and ABTS IC_{50} values 10.88 ± 0.19 µg/mL and 14.47 ± 0.23 µg/mL for hydroxychavicol and 7.66 ± 0.60 µg/mL and 18.00 ± 0.20 µg/mL for eugenol, separately. The major phenolic compound hydroxychavicol antioxidant activity is closer to ascorbic acid than eugenol.

Sazwi et al. (2013) has been previously reported the antioxidant DPPH IC_{50} of betel leaf was 179.5 ± 93.1 μg/mL, which matched our determined DPPH IC_{50} values. So we can say our analysis is justified. $ABTS^{\bullet+}$ assay is used for both lipophilic and hydrophobic antioxidant systems. In contrast, DPPH is used in the case of the lipophilic antioxidant system (Kim et al., 2002). The DPPH IC_{50} and ABTS IC_{50} value of betel leaf extract is shown in Table 4.2. Most IC_{50} values show slightly more $ABTS^{\bullet+}$ scavenging activity than the DPPH scavenging activity when ascorbic acid was used as a standard in both cases. Our findings are in agreement with the previous results reported (Khanam et al., 2012), which shows lower antioxidant capacities for most leaf vegetables equivalent to quercetin, trolox, and ascorbic acid after comparison of DPPH assay to the ABTS assay.

Table 4.2. Antioxidant (IC_{50}) values in FBL, hydroxychavicol, eugenol, and ascorbic acid

Sample name	DPPH IC_{50}	ABTS IC_{50}
Raw extract (mg/mL, dry weight basis)		
Piper betle L. var Bangla	0.43 ± 0.01	0.11 ± 0.01
Piper betle L. var. Sanchi	0.33 ± 0.01	0.10 ± 0.01
Piper betle L. var. Misti	0.32 ± 0.01	0.07 ± 0.00
Piper betle L. var. Khasia	0.17 ± 0.01	0.04 ± 0.00
Piper betle L. var. BARI Paan 3	0.17 ± 0.01	0.04 ± 0.00
Pure compound (μg/mL)		
Ascorbic acid	9.12 ± 0.90	5.14 ± 0.50
Hydroxychavicol	10.88 ± 0.19	7.66 ± 0.60
Eugenol	14.47 ± 0.23	18.00 ± 0.20

Mean ± standard deviation (n = 3), 0.00 represents the value less than 0.01

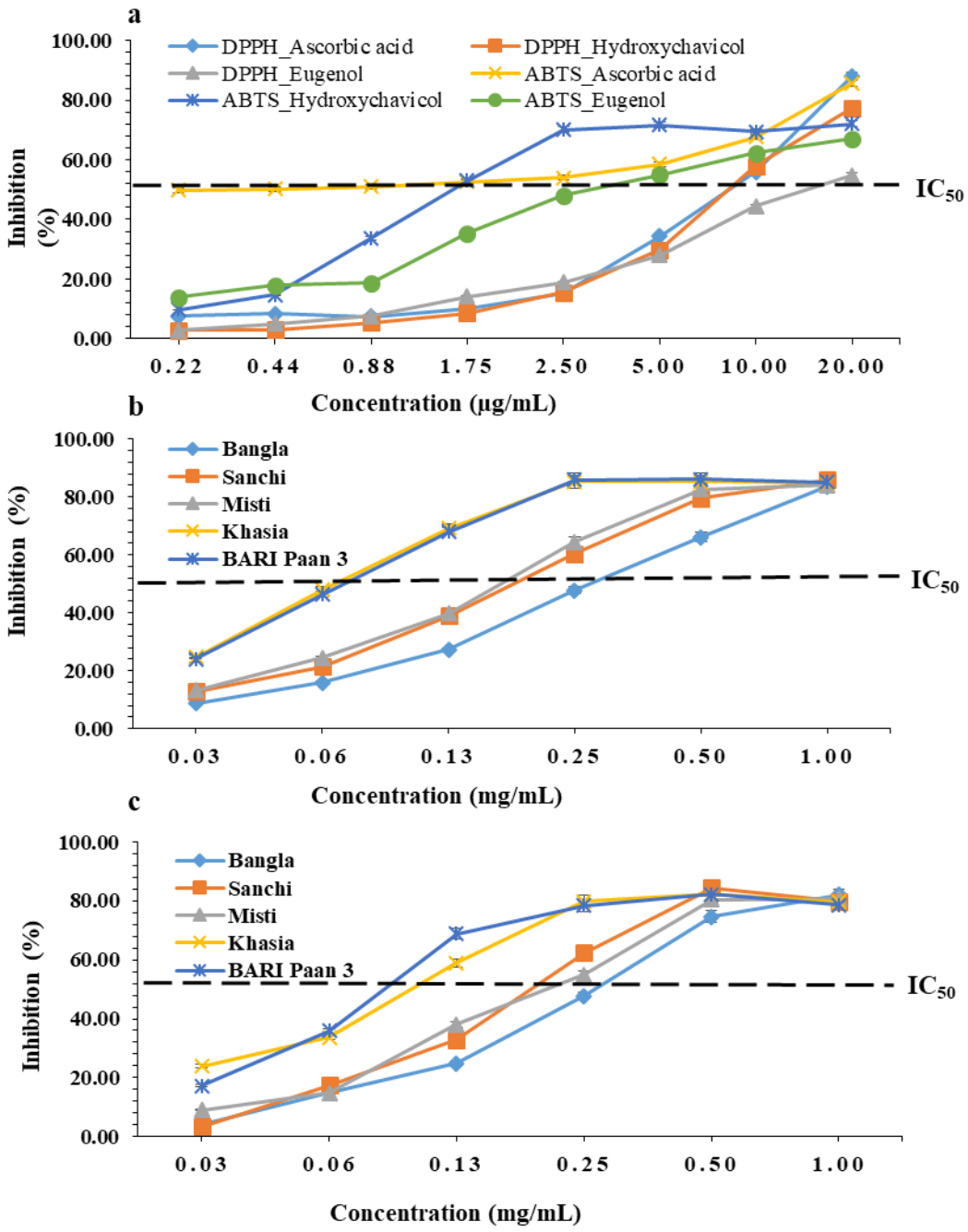


Figure 4.1. Comparison of antioxidant activity in FBL hydroxychavicol, eugenol, and ascorbic acid; (a = DPPH and ABTS assay of ascorbic acid, hydroxychavicol and eugenol, b = DPPH assay in FBL, c = ABTS assay in FBL).

4.3.2. Correlation among total phenol, hydroxychaviol, eugenol versus antioxidant activity

Quantitative determination of total phenolic content, DPPH and ABTS results is greatly influenced in FBL and their major phenolic compound (hydroxychavicol and eugenol). Hence, correlation analyses between the studied parameters were analyzed within the extracts of each variable. FBL with their major phenolic compound hydroxychavicol shows a strong correlation between studied parameters when analyzed separately. In Figure 4.2 (a-f) displays the relationship between different parameters in FBL. The coefficient of determination (R^2) obtained from linear regression analyses between these two parameters were in a range of 0.988 to 0.4405, indicating the negative to the positive correlation. The positive correlation (R^2 = 0.9422) between total phenol content and major phenolic compound hydroxychavicol in Figure 4.2. (a). A significant difference in FBL with a strong positive correlation was observed. A weak negative relation is showed between eugenol versus DPPH Figure 4.2. (b). According to Figure 4.2 (c, d, e, and f), DPPH and ABTS assays exhibit a significant negative correlation with total phenol and hydroxychavicol. Overall, it is concluded that the increase in total phenol may be related to the increase in antioxidant activities.

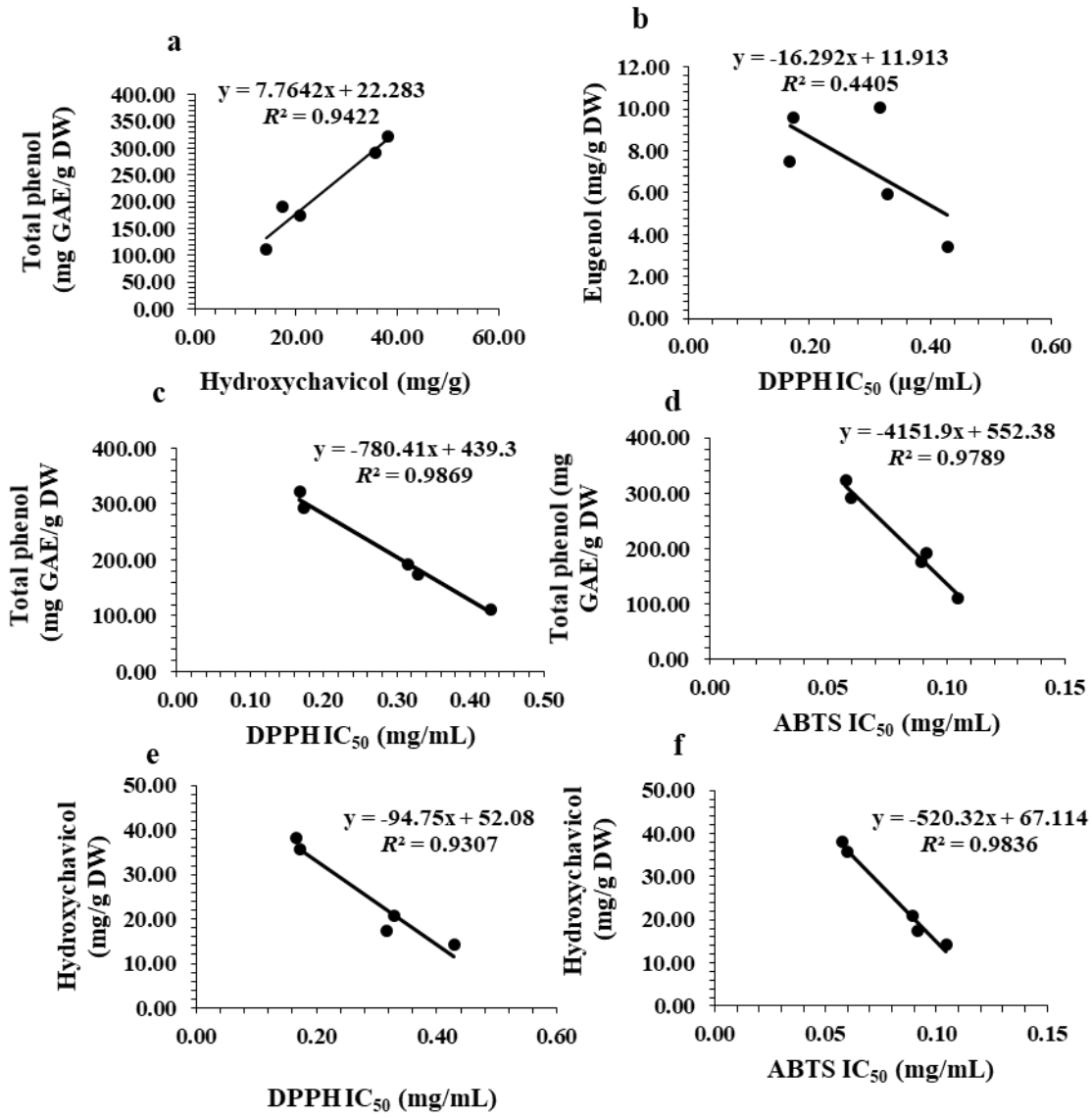


Figure 4.2. (a-f): Effect on different parameter analysis on the correlation between in FBL from Bangladesh (a = total phenol versus hydroxychavicol, b = eugenol versus DPPH IC_{50}, c = total phenol versus DPPH IC_{50}, d = total phenol versus ABTS IC_{50}, e = hydroxychavicol versus DPPH IC_{50}, f = hydroxychavicol versus ABTS IC_{50}).

4.3.2. Antibacterial activity

Table 4.3. shows the antibacterial activity in terms of zone of inhibition (mm in diameter) in FBL extract with two pure compounds (hydroxychavicol and eugenol) and one antibiotic (ciprofloxacin). To our knowledge, there is no antimicrobial study on optimized ultrasonic ethanolic extract in FBL from Bangladesh and their major phenolic compound hydroxychavicol (non-volatile organic compound) and eugenol (volatile organic compound). Our analysis of the extracts producing growth-inhibitory zone ≥ 10 mm in disc diffusion assay was selected as antimicrobial activity. In this study, the tested betel leaf extract and their major phenolic compounds have more significant antimicrobial activity against four gram-negative bacteria (*S. typhimurium, E. coli, P. aeruginosa, A. faecalis*) and two gram-positive bacteria (MRSA, *and M. smegmatis*) which are responsible for different foodborne diseases. One antibiotic (ciprofloxacin) was used as a positive control to compare with FBL with hydroxychavicol and eugenol. One gram-negative bacteria (ESBL) and four gram-positve bacteria (*M. luteus, S. aureus,* VRSA *staphylococcus*) were not showed antibacterial activity. According to Table 4.3. and Figure 4.3, three bacteria (*S. typhimurium*, MRSA*, and M. staphylococcus*) have a greater zone showed in Khasia and BARI Pann 3 than Bangla, Sanchi, and Misti.

Some studies have evaluated the antibacterial activity of betel leaf (Avijit et al., 2020; Agarwal et al., 2012). Gram-negative bacteria are considered to be more resistant due to their outer membrane which acting as a barrier to many environmental substances, including antibiotics (Ratledge and Wikinson, 1998). This outer membrane includes the asymmetric distribution on the lipids with phospholipids and lipopolysaccharide (LPS) located in the inner and outer leaflets, respectively. These characteristics that are absent in the gram-positive bacteria might have acted as the additional barrier that hinders the movement of a foreign substance into the cell (Pagès et al., 2008). Gram-negative bacteria pathogen was more resistant than gram-positive bacteria because of distinctive composition, morbidity, and mortality worldwide (Breijyeh et al., 2020). The results of this study show that the optimized ultrasonic betel leaf

extract exhibited appreciable antibacterial properties which inhibiting the growth of more gram-negative bacteria than gram-positive bacteria. It corroborated the previous reports that the plant extracts are more active against gram-negative bacteria (Agarwal et al., 2012). It could serve as a useful source of potential antimicrobial agents for gram-negative resistance bacteria.

Table 4.3. Antimicrobial assay in FBL, hydroxychavicol, eugenol, and ciprofloxacin

Microorganisms	Zone of inhibition (mm)							
	Bangla	Sanchi	Misti	Khasia	BARI Paan 3	Hydroxy chavicol	Eugenol	Ciproflo xacin
Gram-negative bacteria								
Escherichia coli KCTC 1923	15.00 ± 1.00	14.33 ± 0.58	19.33 ± 1.15	22.33 ± 0.58	21.67 ± 0.58	13.67 ± 0.58	11.67 ± 0.58	20.67 ± 1.15
Pseudomonas aeruginosa KCTC 1637	12.33 ± 0.58	15.67 ± 0.58	15.67 ± 0.58	19.33 ± 1.15	16.00 ± 0.00	11.00 ± 1.00	12.00 ± 0.00	45.33 ± 1.15
Salmonella typhimurium KCTC 1925	25.33 ± 1.15	23.33 ± 1.15	27.67 ± 0.58	29.33 ± 1.15	28.67 ± 1.15	23.33 ± 1.15	25.33 ± 1.15	39.33 ± 1.15
Alcaligenes faecalis ATCC 1004	12.00 ± 0.00	13.67 ± 0.58	16.00 ± 1.00	18.00 ± 0.00	18.00 ± 0.00	11.67 ± 0.58	12.00 ± 0.00	44.67 ± 1.53
Extended-spectrum beta-lactamase W1	-	-	-	-	-	-	-	12.00 ± 1.00
Gram-positive bacteria								
Methicillin-resistant Staphylococcus aureus B15	18.00 ± 1.00	19.67 ± 0.58	24.33 ± 0.58	27.67 ± 0.58	27.67 ± 0.58	19.33 ± 1.15	20.00 ± 0.00	18.67 ± 1.15
Mycobacterium smegmatis ATCC 9341	8.67 ± 0.58	10.00 ± 0.00	10.00 ± 0.00	10.67 ± 0.58	10.00 ± 0.00	10.00 ± 0.00	11.33 ± 0.58	29.33 ± 1.15
Micrococcus luteus ATCC 9341	-	-	-	-	-	-	12.00 ± 1.00	20.00 ± 1.00
Staphylococcus aureus KCTC 1928	-	-	-	-	-	-	-	18.00 ± 1.00
Bacillus subtilis ATCC 6633	-	-	-	-	-	-	-	12.00 ± 1.00
Vancomycin-resistant Staphylococcus aureus	-	-	-	-	-	-	-	18.00 ± 1.00

Each betel leaf concentration was 100 mg/mL (dry weight basis of the sample), hydroxychavicol, eugenol, and ciprofloxacin concentration was 500 μL/mL

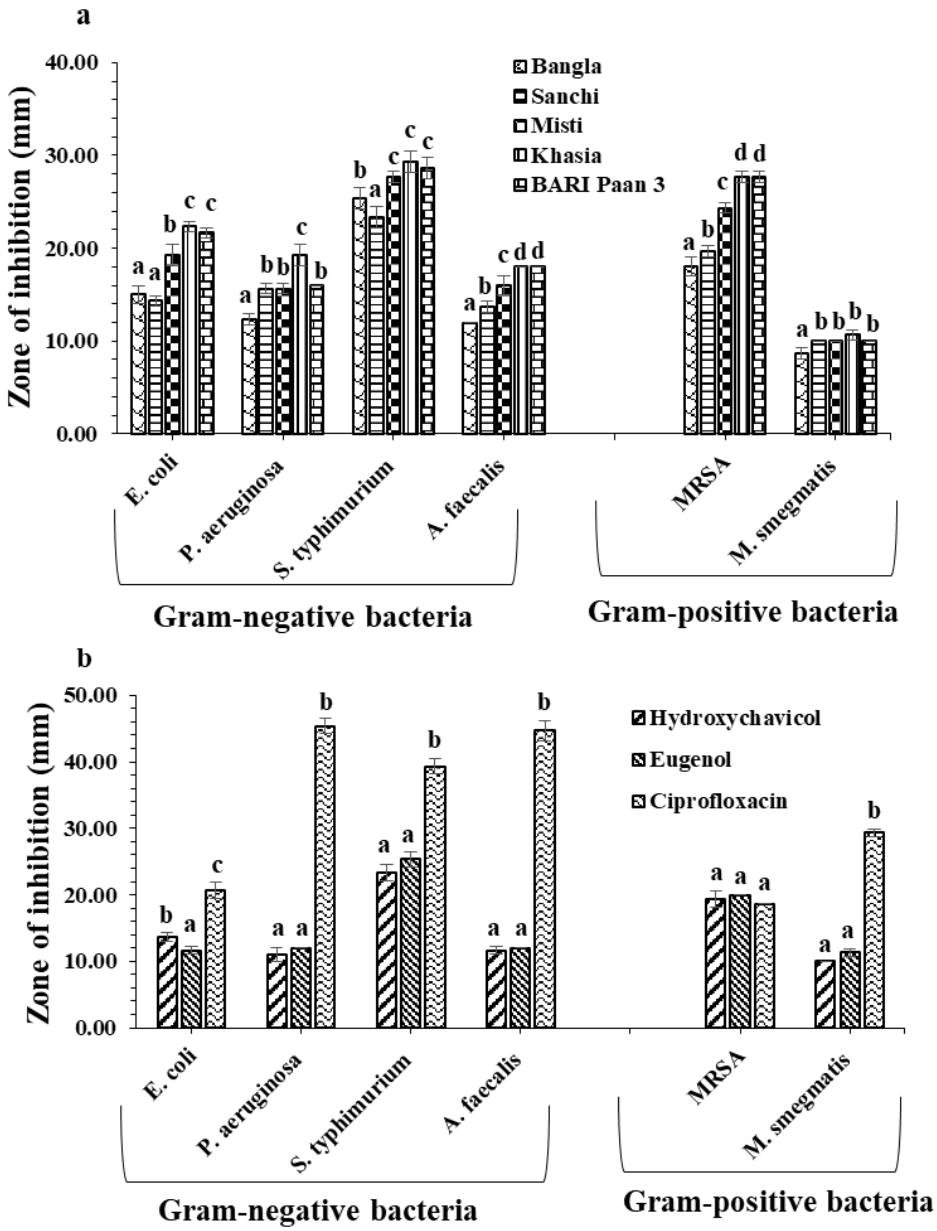


Figure 4.3. Comparison of antimicrobial activity in *Piper betle* L. leaves and standard compound (a) Five varieties of *Piper betle* L. (b) hydroxychavicol, eugenol, and ciprofloxacin. Lowercase letters a, b, c, and d on the bar diagram were used to mark the significance of the difference ($p < 0.05$). Same letters between treatments mean insignificant difference. Different letters between treatments mean significant differences.

4.3.4. Cytotoxicity activity

In this study, the cell viability effect on five varieties of *Piper betle* L. var. (Bangla, Sanchi, Khasia, Misti, and BARI Paan 3) leaf optimized ultrasonic extract from Bangladesh and their two major phenolic compounds, hydroxychavicol (non-volatile organic compound) and eugenol (volatile organic compound) were tested on two cancer cell [human epithelial cell (HeLa) and human neuroblastoma clonal cell (SH-SY5Y)] and one mesenchymal stem cells (MSCs). The cytotoxicity results are graphically showed in Figure 4.4 (a-f). The cell viability was the downward trend with increasing the concentration of all betel leaf extract and test compounds. The 50 % cell viability of HeLa cell (0.19 to 0.32 mg/mL), SH-SY5Y cell (0.41 to 0.50 mg/mL), and MSCs cell (0.46 to 0.96 mg/mL) were determined in FBL. On the other hand, the same procedure 50% cell viability of HeLa cell (3.21 and 3.14 mg/mL), SH-SY5Y cell (6.06 and 5.81 mg/mL), and MSCs cell (28.47 and 32.13 mg/mL), respectively for hydroxychavicol and eugenol were determined (Table 4.4).

The best inhibitory effect was observed at the highest concentration of 0.5 mg/mL for betel leaf extract, and hydoxychavicol and eugenol were 6.01 µg/mL for HeLa SH-SY5Ycells. Among the FBL, BARI Paan 3 was showed the minimum cell viability of 20.66% and 49.00% for HeLa cell and MSCs cell, respectively and Misti paan was 50.42% SH-SY5Y at 0.5 mg/mL. The hydroxychavicol and eugenol showed the minimum 31.47% and 38.62% for Hela cell, 62.08% and 55.63% for SH-SY5Y cell, and 97.92 % and 91.83 % for MSCs cell at 6.01 µg/mL.

The MTT is a positively charged tetrazolium salt, which is reduced by viable cells with an active metabolism, in which it freely enters and forms purple-colored formazan product. MTT assay measures cell viability due to its reductive activity, as it can convert the tetrazolium compound to water enzymatically (Berridge et al., 2005; Kamiloglu et al., 2020). Our result indicates that the HeLa and SH-SY5Y cell, when treated with betel leaf extract and major phenolic compound, shows a significantly low level of cell viability, whereas when MSCs treated with extract and standard solution, they show the non-significant effect of cell viability except

Khasia and BARI paan 3 extracts. Maybe this is due to the lowering of angiogenesis that inhibits the growth of cancer cells. We speculate that the cytotoxicity effect of the betel leaf varieties not only depends on their total phenolic content, total flavonoid, hydroxychavicol, and eugenol concentration, maybe another compound have factor to cell viability of betel leaf extract.

Plant-derived natural compounds have played a vital role as clinically useful anticancer agents (Islam et al., 2009; Ciardiello et al., 2000). Comprehensive experimental studies have been conducted in recent years to establish new antitumor agents that can selectively inhibit significant pathways that regulate the proliferation of the cancer cell (Ciardiello et al., 2000). The effect was much greater on cancer cells than on normal cells. This suggests a selective toxic effect on the cancer cells of the betel leaf extract. It was a general discovery in both cell lines that the cells grown as suspension were relatively more susceptible to the adherent cell types. The letter required a higher extract concentration to bring about the same cell death rate. It was demonstrated by the fact that the 50 % cell viability were lower in HeLa and SH-SY5Y cells than MSCs cells. Normal cells had much higher IC_{50} values, which suggested that the extract had a lower toxic effect on them.

In-plant secondary metabolites, irregular occurring compounds that characterize certain plants or plant groups, a promising new source of therapeutic agents has been identified (Scheck et al., 2006). Several phenolic compounds, including chevibetol, allylpyrocatechol, hydroxychavicol, dotriacontanoic acid, β-sitosterol, tritriacontane, and stearic acid, together with their glycosides, has been found in betel leaves (Bhattacharya et al., 2005; Parmar et al., 1998). In this study, the identification of major phenolic compounds hydroxychavicol and eugenol were analyzed by HPLC-DAD and GC-MS, respectively. Polyphenol studies have also provided convincing evidence on the antitumor activity of plant secondary metabolites in different types of cancer (Yang et al., 2001). Many phenolic and flavonoids have been shown to prevent the development of cancer while displaying antioxidant activity in various animal models (Wang et al., 2011; Chang et al., 2002).

Hydroxycahvicol showed anti-proliferative effects on the cell line of oral carcinoma (Chang et al., 2002). By modulating signaling pathways involved in cell functions such as proliferation, cell growth, and differentiation, by influencing the activity of cancer-related enzymes such as cyclooxygenase-2 and phase I or II metabolizing enzymes, or by including cell cycle arrest, antioxidants can inhibit carcinogenesis through other non-antioxidant action (Wang et al., 2011).

In addition, *Piper betle* L. has to possess antioxidant activity besides anticarcinogenic and antimutagenic properties, especially against tobacco carcinogens, due to the presence of phenolic and flavonoids compounds. In comparison to traditional cancer drugs and effective therapeutic means, in this study, hydroxychavicol and eugenol kill cancer cells but normal cells are unaffected. Therefore, a possibility of manufacturing a new anti-cancer drug from betel leaves (Guha and Nandi, 2019).

Table 4.4. Cytotoxicity activity (50 % cell viability) values in FBL, hydroxychavicol, and eugenol

Sample name	**HeLa cell**	**SH-SY5Y**	**MSCs**
Sample (mg/mL, dry weight basis)			
Piper betle L. var. Bangla	0.26	0.44	0.81
Piper betle L. var. Sanchi	0.26	0.44	0.96
Piper betle L. var. Misti	0.32	0.41	0.92
Piper betle L. var. Khasia	0.22	0.54	0.46
Piper betle L. var. BARI paan 3	0.19	0.50	0.47
Standard (μg/mL)			
Hydroxychavicol	3.21	6.06	28.47
Eugenol	3.14	5.81	32.13

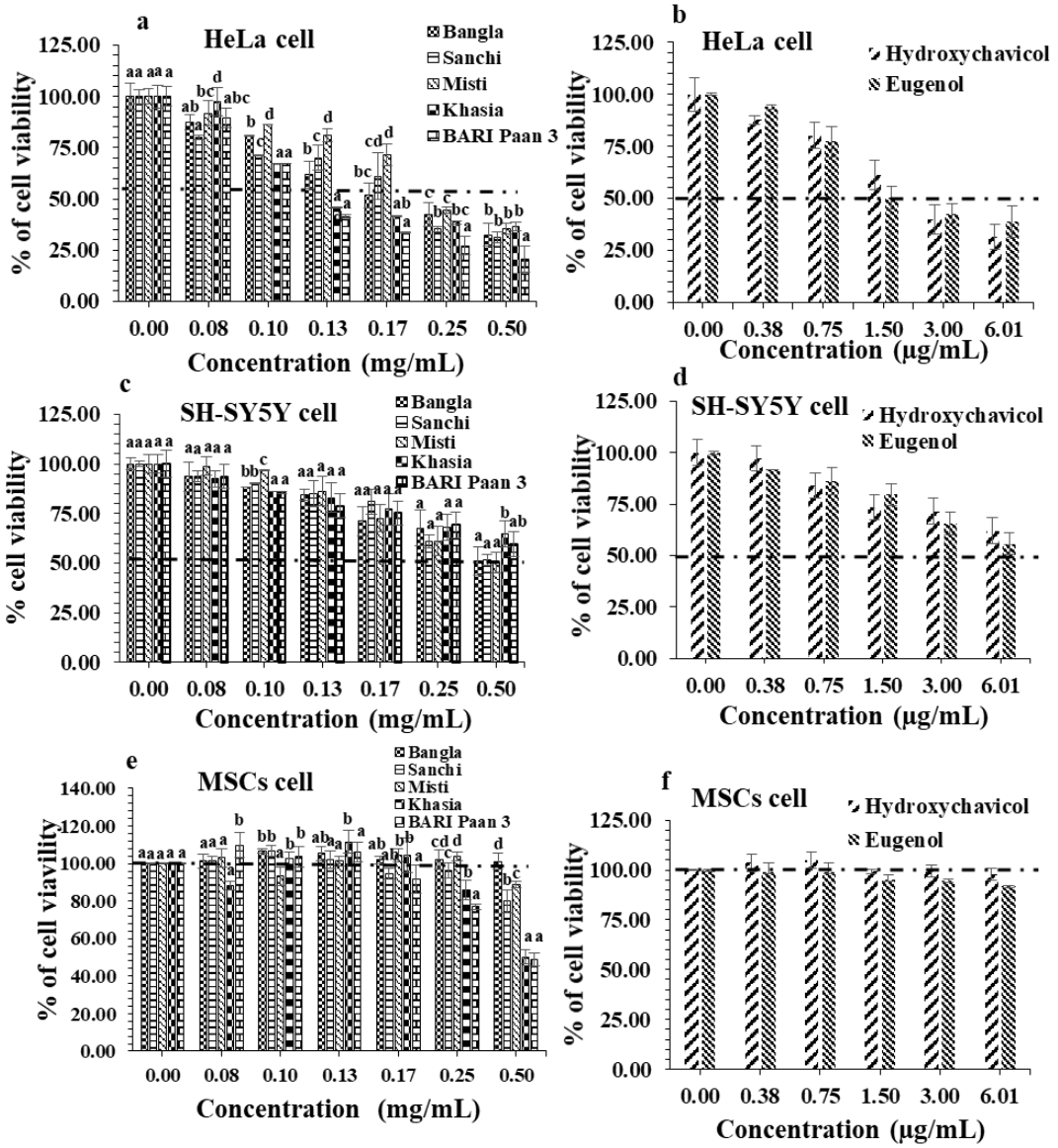


Figure 4.4 (a-f). Comparison of MTT assay showing the percentage of cell viability in FBL, hydroxychavicol, and eugenol; (a and b) HeLa cells (c and d) SH-SY5Y cells (e and f) MSCs Cells. Lowercase letters a, b, c, and d on the bar diagram were used to mark the significance of the difference ($p < 0.05$). Same letters between treatments mean insignificant difference. Different letters between treatments mean significant differences.

4.4. Conclusion

In the fourth part, the comparative biochemical (antioxidant, antimicrobial, and cytotoxicity) properties were determined in FBL from Bangladesh with their major phenolic compounds hydroxychavicol and eugenol. The optimized ultrasonic FBL extract and two pure compounds (hydroxychavicol and eugenol) were used in this analysis. The antioxidant, antimicrobial, and cytotoxicity activity showed different quantity in the FBL, based on the overall results. The antioxidant activity of DPPH IC_{50} and ABTS IC_{50} assay was ranged from 0.11 ± 0.01 to 0.4 ± 0.01 µg/mL and 0.04 ± 0.00 to 0.11 ± 0.01 µg/mL, respectively in the FBL extract. The optimized ultrasonic extract of FBL, hydroxychavicol and eugenol displayed good inhibitory properties against *S. typhimurium*, *S. typhimurium*, and MRSA bacteria compared to other tested bacterias. Khasia and BARI Paan 3 showed more antioxidant and antimicrobial activity than other three varieties (Bangla, Sanchi, and Misti). Additionally, cytotoxicity was assessed on human cancer cell lines and found that the extracts of BARI Paan 3 and Misti showed the highest kill for cancer cells (HeLa and SY-HS5Y), respectively. In contrast, normal cells (MSCs) were not affected by them. In a nutshell, betel leaf extract and its major phenolic compounds hydroxychavicol and eugenol could be the potential anticancer agents.

SUMMARY

This study was performed to evaluate five varieties of *Piper betle* L. (FBL) var. Bangla, Sanchi, Misti, Khasia, and BARI Paan 3 from Bangladesh based on their chemical (elements; major, minor, trace, and toxic; volatile organic compounds and non-volatile organic compounds) and biochemical (antioxidant, antimicrobial, and cytotoxicity activities) properties. Modern instrumental techniques (ICP-OES, ICP-MS, SDE/GC-MS, and HPLC-DAD) were employed to analyze betel leaf varieties.

ICP-OES and ICP-MS analysis of mineral concentrations in FBL were good sources of major, minor, and essential trace elements. The target subject species were found to be contributing good nutritional values of potassium, calcium, zinc, manganese, and copper to the overall intake of consumers. According to the WHO and FAO, the concentration ranges of all analyzed elements were detected within the safe limit except Pb and Mn. From the multivariate chemometric analysis, the PCA and HCA for the application of all analyzed elements were successfully separated and classified into three clusters such as Sanchi formed cluster I; Bangla and Misti formed cluster II; BARI Paan 3 and Khasia formed cluster III.

Simultaneous distillation extraction with *n*-pentane and diethyl ether solvent was used to extract volatile compounds from FBL, resulting in 50 more volatile compounds than published literature. GC-MS analysis showed remarkable differences in the amount of volatile compounds among subject leaves of betel varieties. The highest amount of volatile compounds was found in the Misti betel leaf (13958.90 mg/kg), while the lowest amount was found in the Bangla betel leaf (4346.84 mg/kg). Eugenol was present in the highest amount in all varieties, with the peak area varying from 67.73 % to 85.18 %. The other major volatile compounds found across all varieties of betel leaves were β-caryophyllene, valencene, γ-muurolene, chavicol, and caryophyllene oxide. Through the PCA and HCA analysis, the FBL were separated and classified into three clusters based on their volatile compounds identified in the current study such as, Misti

and BARI Paan 3 formed cluster I; Bangla formed cluster II; and Khasia and Sanchi formed cluster III.

The phenolic compound is higher than other types of phytochemical classes in FBL. For the optimization of ultrasonic extract, the solvent was selected as ethanol: acetic acid: water (70%: 5%: 25%, v/v) and the optimal extraction condition was: time, 90 min; extraction temperature, 75 °C; solid/liquid ratio, 1:15.41. In comparison, the maximum amount of total phenol was in Khasia betel leaf (322.80 mg GAE/g DW) and the minimum amount in Bangla betel leaf (110.51 mg GAE/g DW). The hydroxychavicol quantity was highest in Khasia (38.19 ± 0.37 mg/mL) and lowest in Bangla (14.19 ± 0.31 mg/mL) betel leaves.

The FBL and their major phenolic compound hydroxychavicol and eugenol showed good antioxidant activity compared to ascorbic acid and displayed good inhibitory properties against *S. typhimurium* and *M. staphylococcus* and *M. R. S. aureus* bacteria compared to other tested bacteria. Khasia and BARI Paan 3 has shown more antioxidant and antimicrobial activity than the other three varieties (Bangla, Sanchi, and Misti). Additionally, cytotoxicity was assessed on human cancer cell lines and found that the extracts of BARI paan 3 and Misti were showed the highest cell viability in HeLa and SY-HS5Y cancer cells, respectively. In contrast, normal cells (MSCs) are not affected by betel leaf extract with hydroxychavicol and eugenol.

In conclusion, the selected FBL were good sources of essential elements for example, potassium, calcium, zinc, manganese, and copper. These leaves also rich in sources of volatile organic compound (eugenol) and non-volatile compound (hydroxychavicol) with good antibacterial effect against *S. typhimurium*, *M. staphylococcus*, *E. coli*, *A. faecalis*, and MRSA bacteria and kill the cancer cell (HeLa and SY-HS5Y). At the same time, normal cells (MSCs) were not affected. Finally, we can say betel leaf is an important medicinal herb for eating. There is no adverse health effect based on mineral elements, volatile and non-volatile organic compounds, antimicrobial, and cytotoxicity activity in FBL from Bangladesh.

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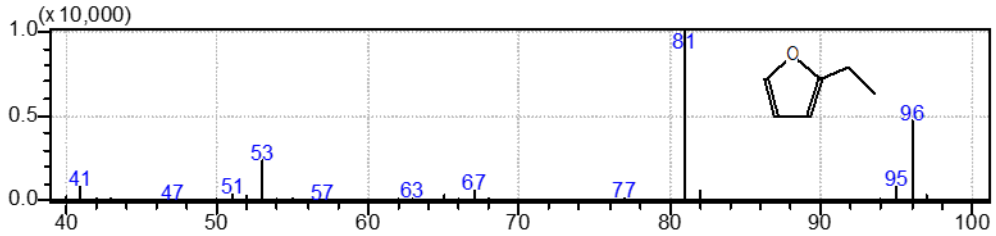
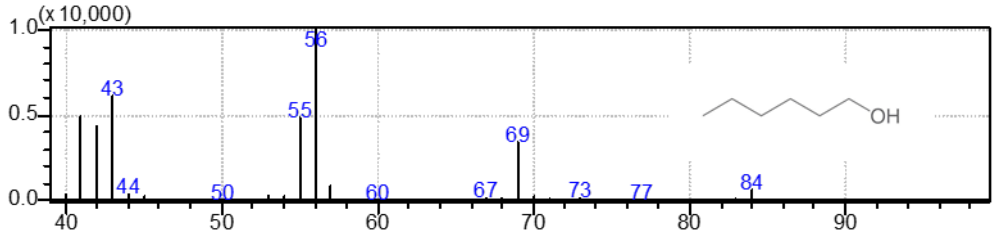
Appendix I: Mass spectra of some major bioactive volatile compounds identified in five varieties *Piper betle* L. from Bangladesh

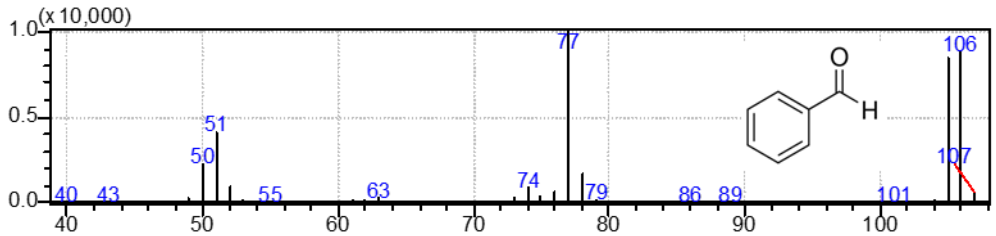
2-Ethylfuran



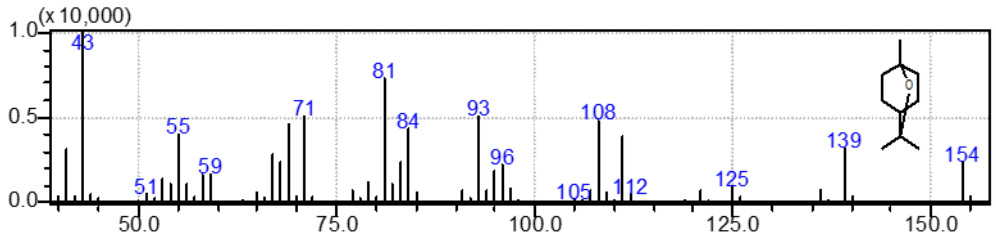
Hexanol



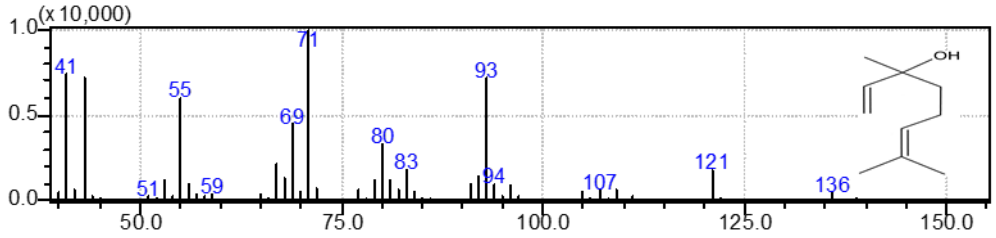
Benzaldehyde



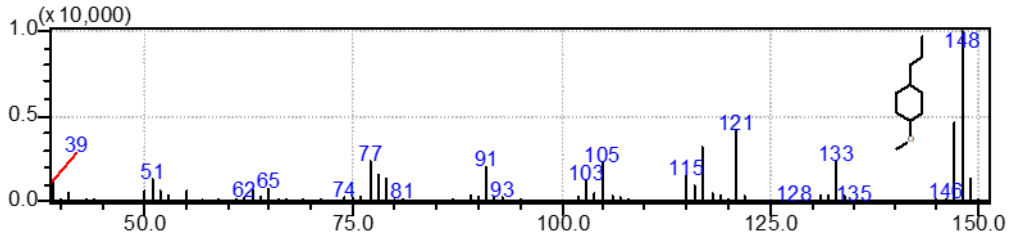
Eucalyptol



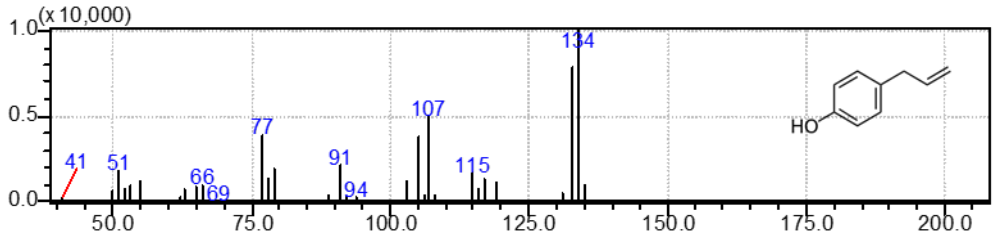
Linalool



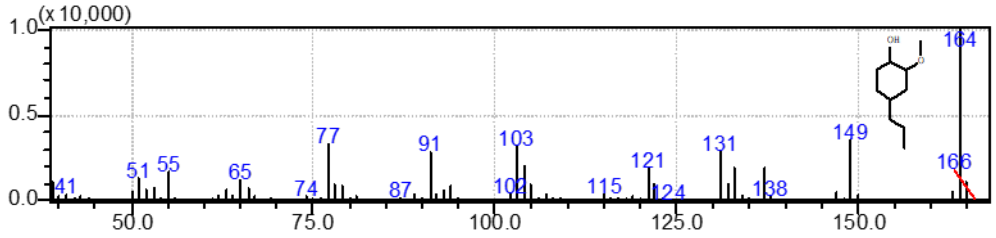
Estragole



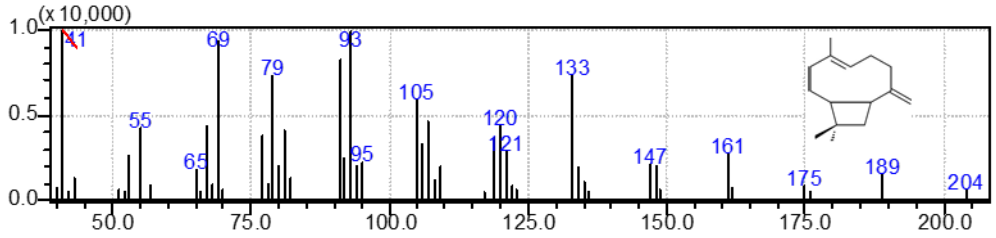
Chavicol



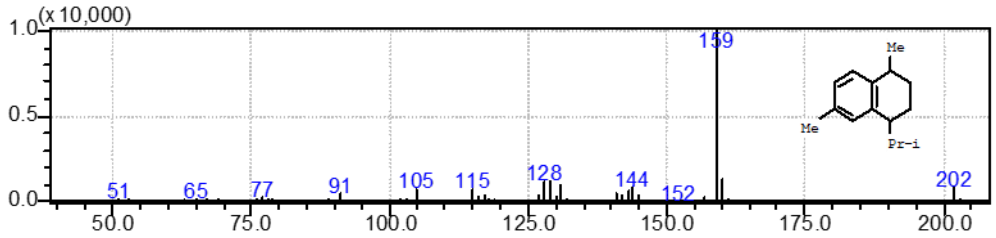
Eugenol



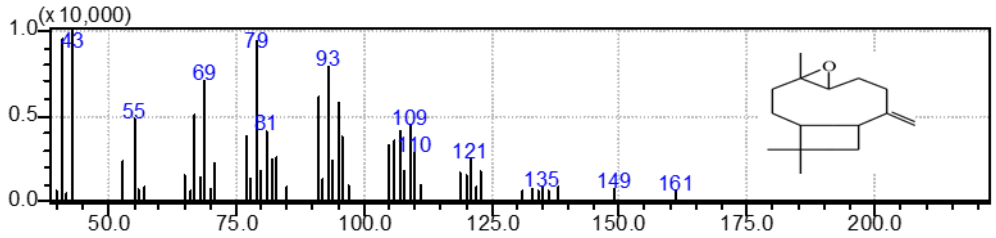
β-caryophyllene



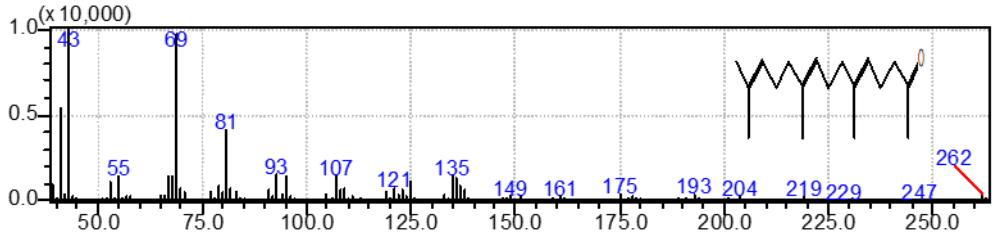
(*E*)-calamenene



Caryophyllene oxide



Farnesyl acetone



Curriculum Vitae
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1984	:	Born on December 15; at Village: North Krishnopur; Thana: Phulbari; District: Dinajpur-5200; Bangladesh
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2018-till now	:	**Ph.D.** study under the supervision of Professor Kim Kyong Su, Ph.D., Department of food and nutrition, Chosun University, Gwangju, Republic of Korea. (**Student ID:20187793**)
August 2021		Final examination to get Ph.D. degree

List of journal articles publication during Ph.D.

1) Young Shin Hong, Ji Young Jeong, Ji Hyeon Son, Ok Yeon Song, Hwang In Min, **Md. Atikul Islam**, Hui Eun Kim & Kyong Su Kim, Changes of Inorganic Component in Bone Broth of Hanwoo Cow by Extraction Time, *Korean Journal of Food and Cookery Science*, 35 (5), 460-470, 2019.
2) **Md Atikul Islam**, Keun Young Ryu, Naeem Khan, Ok Yeon Song, Ji Young Jeong, Ji Hyeon Son, Nargis Jamila & Kyong Su Kim, Determination of the Volatile Compounds in Five Varieties of *Piper betle* L. from Bangladesh Using Simultaneous Distillation Extraction and Gas Chromatography/Mass Spectrometry (SDE-GC/MS), *Analytical Letters*, 53 (15) 2413–2430, 2020.
3) **Md Atikul Islam**, In Min Hwang, Naeem Khan, Ok Yeon Song, Ji YoungJeong, Ji Hyeon Son, Nargis Jamila & Kyong Su Kim, Authentication of Leaves and Petioles of *Piper betle* L. Varieties via Elemental Composition and Multivariate Chemometric Analysis Authentication of Leaves and Petioles of *Piper betle* L. Varieties via Elemental Composition and Multivariate Chemometric Analysis, *Analytical Letters*, *54* (11) 1794-1808, 2021.
4) Ok Yeon Song, **Md Atikul Islam**, Ji Hyeon Son, Ji Young Jeong, Hui Eun Kim, Lee Seung Yeon, Naeem Khan, Nargis Jamila & Kyong Su Kim, Elemental composition of pork meat from conventional and animal welfare farms by inductively coupled plasma-optical emission spectrometry (ICP-OES) and ICP-mass spectrometry (ICP-MS) and their authentication via multivariate chemometric analysis, *Meat Science*, 172 (2) 108344-108350, 2021.
5) Ji Hyeon Son, **Md Atikul Islam**, Joon Ho Hong, Ji Young Jeong, Ok Yeon Song, Hui Eun Kim, Naeem Khan, Nargis Jamila & Kyong Su Kim, Extraction of Volatile Organic Compounds from Leaves of *Ambrosia artemisiifolia* L. and Artemisia annua L. by Headspace-Solid Phase Micro Extraction and Simultaneous Distillation Extraction and Analysis by Gas Chromatography/Mass Spectrometry, *Food Science and Biotechnology*, *30* (3) 355 -366, 2021.

6) Ji Young Jeong, **Md Atikul Islam**, Joon Ho Hong, Ji Hyeon Son, Ok Yeon Song, Naeem Khan, Nargis Jamila & Kyong Su Kim, Determination of Bioactive Marker Compounds to Discriminate *Syneilesis palmata* and *Paris verticillata* Leaves Using High Performance Liquid Chromatography-Diode Array Detector (HPLC-DAD) and Liquid Chromatography Mass Spectrometry-Ion Trap- Time of Flight (LCMS-IT-TOF), *Analytical Letters*, Taylor & Francis, 2021, doi.org/10.1080/00032719.2021.1878527

List of conference oral presentations during Ph.D.

1 Jiyeon Choi, **Md. Atikul Islam** & Kyong Su Kim, Qualitative and Quantitive Analysis of Adulteration in Korean Red Ginseng (Panax ginseng) Commercial Products Using HPLC and LC-MS, Green Chemistry Conferences, Spring 2019 National Meeting & Exposition, American Chemical Society, AGFD 12, March 31 to April 4th, 2019, Orlando, **Florida, USA**.

2. **Ph.D. competition with the best award**

Md. Atikul Islam & Kyong Su Kim, Extract optimization, Total Phenol, Total Flavonoid and HPLC-DAD Analysis with Evaluation their Antioxidant, Antimicrobial, and Cytotoxicity Activity in Five Varieties of *Piper betle* L. Leaves from Bangladesh, 2020 KFN international symposium and annual meeting, OP04, page 21, October 21-23, 2020, **Jeju, Korea**.

3. **As a Young Scientist presentation**

Md. Atikul Islam & Kyong Su Kim, Optimization of Ultrasound-Assisted Extraction of Total Phenol, Antioxidant, Antimicrobial and HPLC-DAD Analysis in Five Verities of Betel Leaves from Bangladesh and Preparation and Characterization of Chitosan Film Incorporated with Betel Leaf Extract, The 30th annual meeting & international symposium KSCC, 2020, Sunchon National University, December 3-4, 2020, **Sunchon, South Korea**.

List of conference poster papers during Ph.D.

1) Hui Eun Kim, **Md Atikul Islam**, Ji Young Jeong, Seung Yeon Lee, Su Mi Park & Kyong Su Kim, Comparison of volatile organic compounds in *Perilla frutescens* var. viridis Makino by simultaneous steam distillation extraction and solid-phase micro extraction, The 30th annual meeting & international symposium KSCC, 2020, PP-37, Page 67, December 3-4, 2020, **Sunchon, South Korea**.
2) Seung Yeon Lee, Hui Eun Kim, Ji Young Jeong, **Md Atikul Islam** & Kyong Su Kim, Determination the status of lactose and gluten foods, The 30th annual metting & international symposium KSCC, 2020, PP-36, Page 66, December 3-4, 2020, **Sunchon, South**.
3) Seung Yeon Lee, Ji Young Son, Ok Yeon Song, Hui Eun Kim, Ji Young Jeong, **Md Atikul Islam** & Kyong Su Kim, Analysis the Status about Lactose and Gluten Foods, 2020 KFN international symposium and annual meeting, P-01-42, page 31, October 21-23, 2020, **Jeju, Korea**.
4) Hui Eun Kim, **Md Atikul Islam**, Ji Young Jeong, Seung Yeon Lee, Su Mi Park & Kyong Su Kim, Comparison of Simultaneous Steam Distillation Extraction and Solid-Phase Micro Extraction for the Analysis of Volatile Organic Compounds in *Perilla frutescens* var. viridis Makino, 2020 KFN international symposium and annual meeting, P-01-41, page 31, October 21-23, 2020, **Jeju, Korea**.
5) Hee Eun Kim, Ok Yeon Song, **Md. Atikul Islam**, Ji Young Jeong, Ji Hyeon Son & Kyong Su Kim, Comparison of Macro and Micro Elements between Animal Welfare and Non-Welfare Chicken by ICP-OES & ICP-MS, KoSFoP 2020 41th International Symposium & Annual Meeting, P3-30, page 124, August 19-21, The Ocean Resort, **Yeosu, South Korea**.
6) Ji Young Jeong, Joon Ho Hong, Ji Hyeon Son, **Md. Atikul Islam**, Ok Yeon Song, Hee Eun Kim & Kyong Su Kim, Determination of the authenticity of *chrysanthemum zawadskii* var. latilobum KITAMURA processed foods using HPLC, KoSFoP 2020 41th International Symposium & Annual Meeting, P3-40, page 125, August 19-21, The Ocean Resort, **Yeosu, South Korea**.
7) Ok Yeon Song, Ji Hyeon Son, Ji Young Jeong, **Md. Atikul Islam**, Hee Eun Kim & Kyong Su Kim, Measurement of hardness and viscosity of aged-friendly foods using

texture analyzer and viscometer, KoSFoP 2020 41th International Symposium & Annual Meeting, P3-31, page 124, August 19-21, The Ocean Resort, **Yeosu, South Korea**.

8) **Md. Atikul Islam**, Ji Young Jeong, Ji Hyeon Son, Ok Yeon Song, Hee Eun Kim & Kyong Su Kim, An optimized ultrasound-assisted method for extraction of total phenolic compounds in *Piper betle* L. from Bangladesh, KoSFoP 2020 41th International Symposium & Annual Meeting, P3-32, page 124, August 19-21, The Ocean Resort, **Yeosu, South Korea**.

9) Hui Eun Kim, Jun Ho Hong, Ji Hyeon Son, Ji Young Jeong, Ok Yeon Song, **Md Atikul Islam** & Kyong Su Kim, Development of analytical method for *perilla frutescens* var. japonica hara and *perilla frutescens* var. acuta kudo using GC-MS, 2020 KOSFST International Symposium and Annual Meeting, P01-107, page 89, July 1-3, **Gwangju, Korea**.

10) Ok Yeon Song, **Md Atikul Islam**, Ji Hyeon Son, Ji Young Jeong, Hui Eun Kim & Kyong Su Kim, Analysis of volatile flavor components in pork meat from conventional and animal welfare farm by SDE/GC-MS, 2020 KOSFST International Symposium and Annual Meeting, P01-106, page 89, July 1-3, **Gwangju, Korea**.

11) Ji Hyeon Son, Ji Young Jeong, Ok Yeon Song, Hui Eun Kim, **Md Atikul Islam** & Kyong Su Kim, Analysis of volatile organic compounds of *ambrosia artemissiifolia* L. extracted by simultaneous steam distillation extraction and solid-phase microextraction, 2020 KOSFST International Symposium and Annual Meeting, P01-105, page 89, July 1-3, **Gwangju, Korea**.

12) Ji Young Jeong, Joon Ho Hong, Ji Hyeon Son, **Md Atikul Islam**, Ok Yeon Song, Hui Eun Kim & Kyong Su Kim, Determination of indicator compound to distinguish between *Perilla frutescens* var. acuta kudo and *Perilla frutescens* var. japonica Hara using HPLC-DAD and LC-MS/MS, 2020 KOSFST International Symposium and Annual Meeting, P01-012, page 63, July 1-3, **Gwangju, Korea**.

13) **Md Islam Atikul**, Ok Yeon Song, Ji Hyeon Son, Ji Young Jeong, Hee Eun Kim & Kyong Su Kim, Volatile Compound Profiling of Two Varieties *Piper betle* L. from Bangladesh, 2019 KFN international symposium and annual meeting, P-01-29, page 18, October 23-25, 2019, **Jeju-Korea**.

14) Ji Young Jeong, Ji Hyeon Son, Ok Yeon Song, **Md Atikul Islam** & Kyong Su Kim, Determination of Total Amount Polyphenol and Flavonoid in *Perilla fructescens* var. japonica Hara and *Perilla fructescens* var. acuta kudo by UV-Vis Spectrophotometer, 2019 KFN international symposium and annual meeting, P-02-77, page 25, October 23-25, 2019, **Jeju-Korea**.

15) Ok Yeon Song, **Md Atikul Islam**, Ji Hyeon Son, Ji Young Jeong & Kyong Su Kim, Comparison of Macro and Micro Elements in Animal Welfare and Non-Welfare Pork by ICP-OES and ICP-MS, 2019 KFN international symposium and annual meeting, P-01-28, page 18, October 23-25, 2019, **Jeju-Korea**.

16) Ji Hyeon Son, Ji Young Jeong, Ok Yeon Song, **Md Islam Atikul** & Kyong Su Kim, Determination of Volatile Organic Compounds in *Perilla fructescens* var. japonica Hara and *Perilla fructescens* var. acuta kudo by Simultaneous Distillation Extraction and GC-MS Analysis, 2019 KFN international symposium and annual meeting, P-01-24, page 18, October 23-25, 2019, **Jeju-Korea**.

17) Ok Yeon Song, Ji Young Jeong, Ji Hyeon Son, **Md Atikul Islam** & Kyong Su Kim, Comparison of Vitamin A and Vitamin E Amount Between Animal Welfare and Non-Welfare Pork by HPLC-DAD, 2019 KFN international symposium and annual meeting, P-02-93, page 26, October 23-25, 2019, **Jeju-Korea**.

18) Ji Hyeon Son, Ji Young Jeong, Ok Yeon Song, **Md Atikul Islam** & Kyong Su Kim, Comparison of Macro Elements in *Perilla fructescens* var. japonica Hara and *Perilla fructescens* var. acuta Kudo by ICP-OES, KOSFOP 2019 40th International Symposium and Annul Metting, P3-3, 124, August 21-23, 2019, **Pyeongchang-Korea**.

19) Ji Yeon Choi, Joon Ho Hong, Eun Yeong Nho, **Atikul Islam** & Kyong Su Kim, Determination of the Inclusion of Non-edible Sardine (*Paris Verticillata*) in the Extract of Umbrella Herb (*Syneilesis palmata*) Using LC-MS / MS, Industrialization of Functional Materials Using Local Specialty Resources, Page 100, P3-18, 15-16 November, 2018, **Gwanju-Korea**.

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Md. Atikul Islam