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PHYTOCHEMICAL STUDY ON BLACK PEPPER BERRIES (*Piper nigrum* L.)

NIROSHA RAMESH[1], AHMAD NAZIF AZIZ[1,2] AND NURUL HUDA ABDUL WAHAB[1,2]*

[1]Faculty of Science and Marine Environment, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Malaysia. [2]Advanced Nano Materials (ANoMa) Research Group, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Malaysia.

**Corresponding author: nhuda@umt.edu.my*
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Abstract: Black pepper is one of the well-known spices rich in aromatic and medicinal components, along with substantial levels of several other functional components that have health-promoting properties. The use of black pepper has been recorded in various fields such as traditional medicine, food processing, and the pharmaceutical industry. It is also recognised as an important source of natural antioxidants that have anticarcinogenic properties. In this study, a chemical investigation using black pepper berries (*Piper nigrum*) was conducted. The purpose of doing that is to determine the chemical component of this species. The sequential maceration extraction methods utilising solvents with different polarities, namely hexane, dichloromethane, and methanol, yielded the corresponding crude extracts. The quantitative phytochemical analysis was performed on the crude extracts. Separation and isolation work was focused on the dichloromethane crude extract using Thin Layer Chromatography (TLC), Column Chromatography (CC), and Preparative Thin Layer Chromatography (PTLC). The outcomes revealed that the berries of *P. nigrum* contained alkaloid, terpenoid, steroid, and flavonoid metabolites. Two semi-pure compounds have been isolated from the dichloromethane crude extract. The obtained compounds were analysed using Fourier Transform Infrared (FTIR) spectroscopy and Ultraviolet-Visible (UV-Vis) spectrophotometer. Based on the FTIR spectrums and previous studies, these semi-pure compounds, K1A (**2**) and K3C (**3**), are expected to be 2, 4-di-tert-butylphenol and ethyl linoleate, respectively. This significant data provides preliminary findings that may lead to further purification and structure determination as a potential source of herbal medicinal compounds.

Keywords: Black pepper, piperaceae, flavour compound, secondary metabolite, chromatography.

Introduction

Piper has a place in the family of Piperaceae, which contains in excess of 1,000 species, and it is broadly disseminated around the tropical areas of the globe (Chaveerach *et al*., 2006). These plants are rich in fundamental oils that are possibly grounded in their natural products: Seeds, leaves, branches, roots and stems. Some Piper species have straightforward substance profiles, while others, for example, *Piper nigrum*, *Piper betle*, and *Piper auritum*, contain extremely different set-ups of secondary metabolites. In customary medication, Piper species were utilised globally to nurse a few infections (Salehi *et al.*, 2019). The major constituents of Piper are mainly the two parts: specifically, volatile oil and pungent mixtures (Gorgani *et al*., 2016).

P. nigrum (black pepper) is a huge agrarian yield with high business, financial, nourishing, well-being, and restorative advantages (Zhu *et al*., 2017; J.S. *et al*., 2018; Zhu *et al*., 2018; Ghodki & Goswami, 2019). The plant is a lasting climbing plant that is locally grown in Southeast Asia and China. Nonetheless, its development is far and wide in tropical locales, going from many pieces of India, Brazil, Indonesia, Sri Lanka, and Vietnam to Malaysia. *P. nigrum* is famous as the spice king due to its pungent quality (Srinivasan, 2007). Indeed, even the worth of pepper is owed to its sharpness and flavour, which is credited to the presence of an alkaloid called piperine (**1**) as an essential oil.

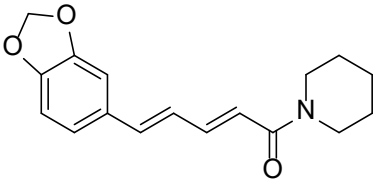

Figure 1: Structure of Piperine (**1**)

The essential oils which make up around 1-7% of dark pepper (Peter, 2012), are accountable for the odour of pepper, although (**1**) exhibits sharpness (Parthasarathy *et al*., 2008). The measure of (**1**) varies in plants having a place with the Piperaceae family; it comprises 2-7.5% of both high-contrast peppers (Peter, 2012; Ravindran, 2012). The (**1**) content of pepper can be affected by many environmental influences (Peter, 2012).

In ancient Chinese and Indian treatment, *P. nigrum* was utilised as a characteristic therapeutic specialist for the therapy and reduction of torment, ailment, flu, solid torments, chills, and fevers. Dark pepper tea was moreover attributed to alleviating head pain, sore throat, indigestion, and unconsciousness (Parthasarathy *et al*., 2008).

Several studies have displayed that (**1**) exhibits antioxidant and chemopreventive activities. Recovered tissues of *P. nigrum,* such as callus, in vitro shoots, roots, in vitro plantlets, peppercorn, and accustomed plantlets, have cell reinforcement movement, which is presumably due to the presence of flavonoids and phenolic substances (Ahmad *et al.*, 2010).

Taking into account the great interest in *P. nigrum* berries, we aim to touch on the chemical composition of these plant extracts, which leads to a new perspective of phytochemical significance.

Materials and Methods

Plant Material

P. nigrum berries (900 grams) were purchased from Subang Jaya, Selangor in July 2020. The samples were cleaned under running tap water and shade dry at room temperature for five days before being pulverised into a powder. The plant powder was then stored in sealed containers at 4°C until further analysis.

Sample Extraction

The *P. nigrum* berries (900 grams) were extracted successively with hexane, dichlorometane, and methanol (each 400 ml x 2) at room temperature using the ultrasonic sonication technique (Elma D-78224 Singen Htw, Germany). The extracts were then concentrated using a rotary evaporator (Büchi Rotavap R-200 CH-9230, Switzerland) under reduced pressure at 35°C - 40°C. The crude extracts obtained were weighed, labelled, and stored in a chiller (4°C).

Qualitative Analysis of Phytochemicals

The phytochemical screening analysis was carried out to determine the presence of secondary metabolite compounds in the crude extract of the *P. nigrum* berries. This work conducted qualitative analysis, which included alkaloids, flavonoids, saponins, and steroids/ triterpenoids, using the following standard procedures (Mukherjee, 2002; Harborne, 2005).

Isolation and Separation

DCM crude extract was pre-adsorbed onto the prepared column. A linear gradient solvent system was performed from 100% hexane/ ethyl acetate/acetone to 100% MeOH, and 40 fractions were collected. The collected fractions were subjected to TLC analysis and managed to have 29 pool fractions based on the R_f value (CF1-CF29). Referring to the TLC profile, CF18 was selected for further separation and purification. Further analysis and a few series of purification (CC and PTLC) led to two semi-pure compounds marked as K1A (**2**) and K3C (**3**).

Results and Discussion

Qualitative Analysis of Phytochemicals

The presence of alkaloids was detected in the *P. nigrum* crude extracts, which is in line with two recent research articles (Gupta *et al.,* 2014; Barkat & Mahmood, 2018). Their study reported that there was a presence of alkaloid compounds in hexane crude extracts. It also revealed that the DCM crude extract of *P. nigrum* possessed a positive result for alkaloids (Ahmad *et al.*, 2020). Besides that, the presence of alkaloids was also detected in the methanolic crude extract (Zahira *et al*., 2016; Anith *et al*., 2018).

P. nigrum crude extracts possessed triterpenoids (Gupta *et al*., 2014; Zahira *et al*., 2016; Barkat & Mahmood, 2018). Moreover, Barkat & Mahmood (2018) also stated that the DCM and methanol crude extracts also have steroid compounds. Gupta *et al*. (2014) reported that DCM crude extract of *P. nigrum* has the presence of lipids/fats, whereas steroids, in particular, are lipids.

Surprisingly, the hexane crude extract of *P. nigrum* indicated the presence of flavonoids (Gupta *et al.*, 2014). Ahmad I (2020) also mentioned that there were flavones in the DCM crude extract as well as Anith *et al*. (2018) and Barkat and Mahmood (2018) discovered a presence of flavone compounds in the methanol crude extract of *P. nigrum*. However, there is no presence of saponins in the crude extracts of *P. nigrum*. This data is particularly in line with the recent studies by Ahmad *et al*. (2020) and Anith *et al*. (2018). Table 1 presents the summaries of the qualitative analysis.

Table 1: Overall results of phytochemical analysis on *P. nigrum* extracts

Sample	Alkaloid Test	Triterpenoid/Steroid Test	Saponin Test	Flavonoid Test
Fresh sample	+	+T	-	+
Hexane crude	+	+T	-	+
DCM crude	+	+T & +S	-	+
Methanol crude	+	+T & +S	-	+

Spectroscopy Analysis of K1A (2)

K1A (**2**) was a semi-pure compound obtained from DCM crude extract. The IR spectrum of (**2**) examines that some speak at certain absorptions. Figure 2 shows a broad absorption peak at 3436.25 cm^{-1} which assesses the presence of hydrogen-bonded OH stretching (Zhuang *et al.*, 2020; Amir *et al.*, 2020). It means that this compound contains a hydroxy group. The strong absorption bands that appeared at 2924.07 cm^{-1} and 2825.62 cm^{-1} were assigned as CH stretching of symmetric and CH stretching of asymmetric, respectively (Ahmad *et al.*, 2020).

Note that the peak determined the C=C stretching aromatic ring was observed at 1640.99 cm^{-1} (Ahmad *et al.*, 2020), while a band absorption at 1465.00 cm^{-1} shows the CH2/CH3 bending deformation (Zhuang *et al.*, 2020) at 908.93 cm^{-1} and a benzene ring out of plane at 720.84 cm-1 (Mistry, 2009). For UV-Vis spectroscopy, (**2**) acetone was used for dilution, and the wavelength was set up at 325 nm - 600 nm while absorbance was at 0 - 0.6A for this analysis.

The absorption indicates the presence of a conjugated system in this compound. (**2**) revealed UV absorption at λmax 330.5 nm and 0.1749A which established the transition of $n \rightarrow \pi^*$ of the conjugated system. Figure 3 illustrates the Ultraviolet-Visible (UV-Vis) spectrum, while Table 2 presents the frequencies and functional groups of (**2**).

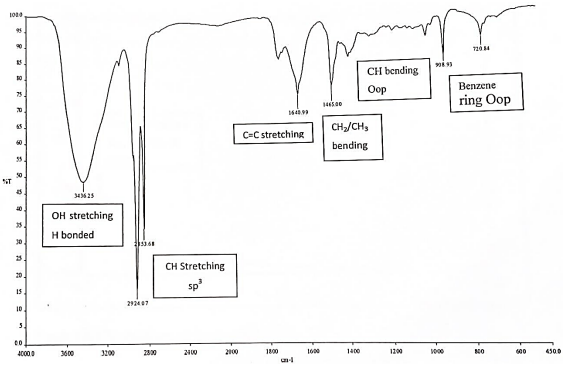

Figure 2: IR Spectrum of K1A (**2**)

Table 2: Frequencies and functional groups of K1A (**2**)

Wavenumber (cm^{-1})	Assignment
3436.25	OH stretching
2924.07	CH stretching (sym) sp^3
2825.62	CH stretching (asym) sp^3
1604.99	C=C stretching
1465.00	CH_2/CH_3 bending
908.93	CH bending Oop
720.84	Benzene ring Oop

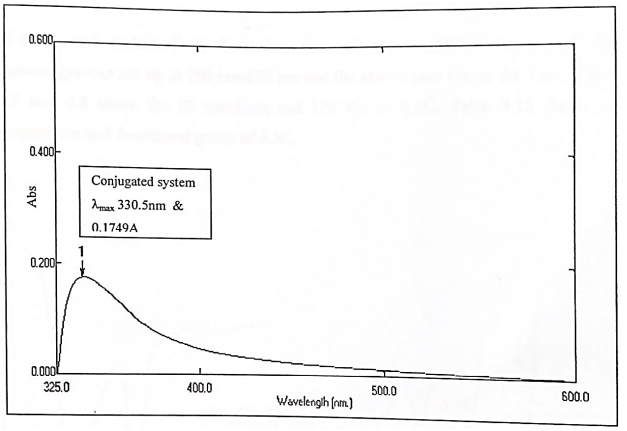

Figure 3: UV spectrum of K1A (**2**)

Compared to the previous studies, (**2**) was a compound containing OH stretching, C=C stretching, CH stretching sp^3, CH3/CH2 bending, and a benzene ring out of the plane. Thus, (**2**) can be predicted as 2,4-di-tert-butylphenol referred to in the previous study listed by Gupta *et al*. (2013). This structure was also proven by a phytochemical test, where it was a positive result for the terpenoid compound. Figure 4 depicts the expected structure of (**2**), known as 2,4-di-tert-butylphenol.

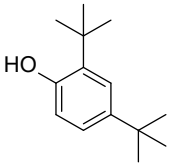

Figure 4: 2,4-di-tert-buthylphenol (**2**)

Spectroscopy Analysis of K3C (3)

The IR spectrum of (**3**) revealed the presence of strong bands at 2920.17 cm^{-1} and 2851.10 cm^{-1} which represent the symmetric and asymmetric of sp^3 CH stretching, which means that K3C contained a long chain of alkyl groups (Paulkumar *et al*., 2014; Ahmad *et al*., 2020). Here, the weak absorption band at 1732.95 cm^{-1} determined the functional group of C=O (Paulkumar *et al*., 2014), while C=C stretching was observed at 1617.28 cm^{-1} (Zhuang *et al*., 2020). The peak at 1445.44 cm^{-1} revealed the presence of C-H deformation (CH2/CH3) (Zhuang *et al*., 2020). Two band absorptions at 1251.32 cm^{-1} and 1038.12 cm^{-1} resemble the C-O stretching (Zhuang *et al*., 2020).

For the UV-Vis spectrum, λmax appears at 298.60 nm with 0.8385A, determining the presence of chromophore C=O, while 336.60 nm with 0.8164A indicated the appearance of a conjugated system. Consequently, both absorptions were in the transition range of $n \rightarrow \pi^*$. The wavelength was set up at 280 nm - 600 nm, and the absorbance was at 0A - 1.0A. Figures 4 and 5 illustrate the IR spectrum and UV-Vis of (**3**), respectively. Table 3 presents the frequencies and functional groups of (**3**).

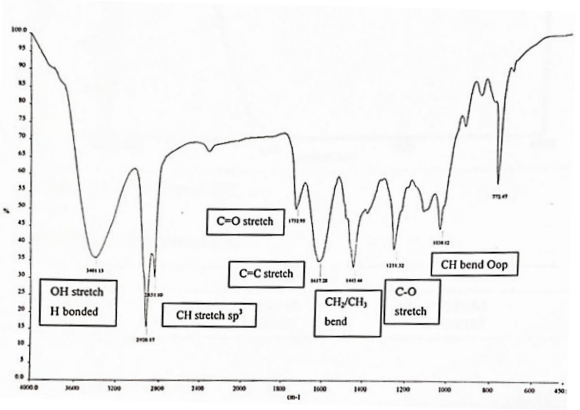

Figure 5: IR Spectrum of K3C (**3**)

Table 3: The frequencies and functional groups of K3C (**3**)

Wavenumber (cm^{-1})	Assignment
3401.13	OH stretching
2920.17	CH stretching (sym) sp^3
2851.10	CH stretching (asym) sp^3
1732.97	C=O stretching
1617.28	C=C stretching
1445.44	CH2/CH3 bending
1251.12	C-O stretching
1038.12	C-O stretching

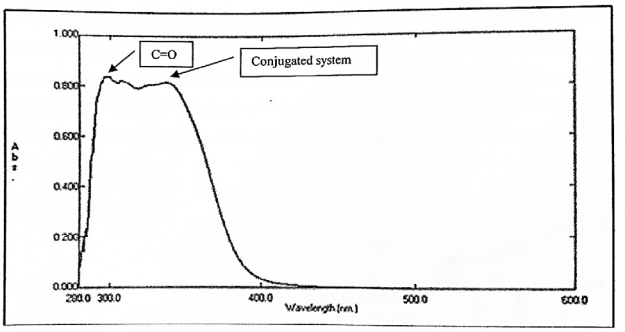

Figure 6: UV Spectrum of K3C (**3**)

(**3**) was predicted as ethyl linoleate, which was listed by Gupta *et al.* (2013), in a previous study. The argument was proven by IR spectroscopy, where some absorptions demonstrated the same functional group as ethyl linoleate, such as OH stretching, C=O stretching, and also C-O stretching. The UV-Vis spectroscopy revealed the chromophore of C=O (~ 298.60 nm) and the conjugated system (~ 336.60 nm), where it can be predicted as ethyl linoleate, as portrayed in Figure 6.

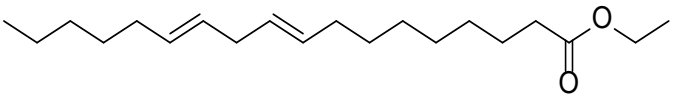

Figure 7: Structure of ethyl linoleate (**3**)

Conclusions

The phytochemical tests indicated the presence of alkaloids, steroids, triterpenoids, and flavonoids in the crude extracts of *P. nigrum*. Two semi-pure compounds, K1A (**2**) and K3C (**3**), need further analysis to confirm the structure. Thus, more analysis on spectroscopy, such as Mass Spectroscopy (MS) and Nuclear Magnetic Resonance (NMR), is strongly recommended.

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