

*Full Length Research Paper*

# Predicting group of metabolites available in partially purified tomato leaves extract showing anticancer activity by high performance liquid chromatography (HPLC) and Fourier transform infrared (FTIR)

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Previously, tomato leaves were proved to be one of the potential anticancer agents. High performance liquid chromatography (HPLC) and Fourier transform infrared (FTIR) spectroscopic instrumentation were used to predict the presence of group of metabolites and to ascertain the possibility of certain absorption bands, in which most of the peaks in partially purified tomato (*Solanum lycopersicon*) leaves methanol extract are attributed to the specific functional groups. The extraction was carried out in a shake flask with 82% methanol, 1:10 (w/v) sample to solvent ratio, agitated at 22°C with 110 rpm within 24 h. Later, the extract was partially purified by column chromatography. HPLC was used to quantify the number of unknown component presents in the fraction. Then, a FT-IR Bruker Tensor 27 System was used during FTIR data acquisition. The collection of FTIR spectra was carried out at 16 scans with resolution of 4 cm<sup>-1</sup> using strong apodization in the frequency regions of 4,000 to 650 cm<sup>-1</sup>. The results support the premise that HPLC and FTIR spectroscopy are efficient and accurate methods for determining major and minor components presents in the extract.

**Key words:** Tomato leaves, anticancer, high performance liquid chromatography (HPLC) and Fourier transform infrared (FTIR).

## INTRODUCTION

Nowadays, tomato (*Solanum lycopersicon*) is one of the most widely consumed fresh vegetables in the industrialized world. It is reported that tomato juice has been used as homemade remedy for oral and prostate cancers (Luckwill, 2003). Lycopene, which is present naturally in tomatoes, has long been identified as an anti-carcinogen. More recent discussions show the positive association between lycopene and health, but also emphasize that there are a family of beneficial compounds in tomato (Laquatra et al., 2005). Besides, lack of efficacy

upon ingestion of purified carotenoid supplements suggests that well-studied carotenoids such as lycopene may act synergistically with other compounds in protecting human health (Ellinger et al., 2006).

According to previous research (Chik et al., 2010), tomato leaves extract also significantly contain bioactive fractions with anticancer properties which can be one of the anticancer agent. This discovery research work is creating a variety of potential new agents worthy of further pursuit as potential therapeutic agents. The most characteristic feature of the development in the methodology of pharmaceutical and biomedical analysis is high performance liquid chromatography (HPLC) (Görög, 2007). This technique has become the most important method in the quality control of bulk drugs, pharmaceutical

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formulations and also in the determination of drugs and metabolites in biological samples. Therefore, this research had applied HPLC to identify and quantify the number of unknown compound presents in the extract. Technically, HPLC is a chromatographic technique that can separate a mixture of compounds, and is used in biochemistry and analytical chemistry to identify, quantify and purify the individual components of the mixture (Chinou, 2011).

Furthermore, the use of Fourier transform infrared (FTIR) spectroscopy is increasing in many fields including drug discovery studies (Guillen and Cabo, 2000). It has been a workhorse technique for materials analysis in the laboratory for over seventy years. An infrared spectrum represents a fingerprint of a sample with absorption peaks which correspond to the frequencies of vibrations between the bonds of the atoms making up the material (Thermo, 2001). Since every different material is a distinctive combination of atoms, no two compounds generate the same infrared spectrum. Therefore, infrared spectroscopy can result in a positive identification of qualitative analysis in different kinds of materials. In addition, the range of the peaks in the spectrum is a direct indication of the amount of compound present. By means of this modern software algorithms, infrared is an outstanding tool for quantitative analysis.

## MATERIALS AND METHODS

### Preparation of tomato leaves methanol extract

Fresh tomato leaves were collected from Cameron Highlands, Pahang, Malaysia and washed using tap water before drying at 40°C for one day in drying oven (Junichiro et al., 2006). The dried leaves were grinded using a grinder machine to increase the surface area. The powdered leaves were then extracted using 82% methanol with 1:10 (w/v) ratio. The mixture was agitated at 22°C, 110 rpm for 24 h in shake flasks. The mixture was filtered with Whatman No.1 filter paper to collect the filtrate. Finally, the filtrate was concentrated in a water bath at 40°C. The extract was dissolved in 10% culture-grade dimethylsulfoxide (DMSO; Sigma-Aldrich, St.Louis, MO, USA) (w/v ratio) for further use.

### Purification by column chromatography

The active fractions were collected and prepared according to Chik et al. (2010) procedures. Slurry of silica gel (60 Å, mesh 200-425, SIGMA, USA) was prepared by mixing 20 g silica powder with 40 ml of ethanol/methanol (6:4) and poured into a clamped column. Then, the eluent was drained from the column before loading the sample. Once the sample was added, the eluent was continuously added while collecting small fractions at the bottom of the column. The purified fractions were collected separately into tubes and were concentrated in a vacuum concentrator

### High performance liquid chromatography

The concentrated sample was dissolved in 82% methanol and the HPLC experiments were performed on Waters 600 Controllers System (USA) with isocratic control. The analysis was carried out in C18 column, 25 cm x 4.6 mm ID (Supelco, USA). The chromatogra-

phic separation was executed with isocratic eluent of acetonitrile / distilled water (3:7), injection volume of 10 µl, constant flow rate and temperature of and 30°C at 1.00 ml/min, respectively. The separation was monitored at 280 nm with a Waters 2998 Photodiode Array Detector. Data were collected and analyzed using a Waters Empower System Software.

### Fourier transform infrared (FTIR) spectroscopy data acquisition

Using a Pasteur pipette, few drops of each dissolved sample were placed in contact with attenuated total reflectance (ATR) on a single bounce plate of ZnSe single crystal at a controlled ambient temperature (25°C). A FT-IR Bruker Tensor 27 System equipped with a room temperature DTGS (deuterated tri-glycine sulfate) detector, mid-IR source (4000 to 400 cm<sup>-1</sup>), a KBr beamsplitter with a maximum resolution of 1 cm<sup>-1</sup> and connected to software of the OPUS (OPTical User Software) operating system (Version 6.0), was used during FTIR data acquisition. The collection of FTIR spectra was carried out at 16 scans with resolution of 4 cm<sup>-1</sup> using strong apodization in the frequency regions of 4,000 to 650 cm<sup>-1</sup>. These spectra were deducted against an air spectrum (background). The results were recorded as absorbance values, and replication was done three times.

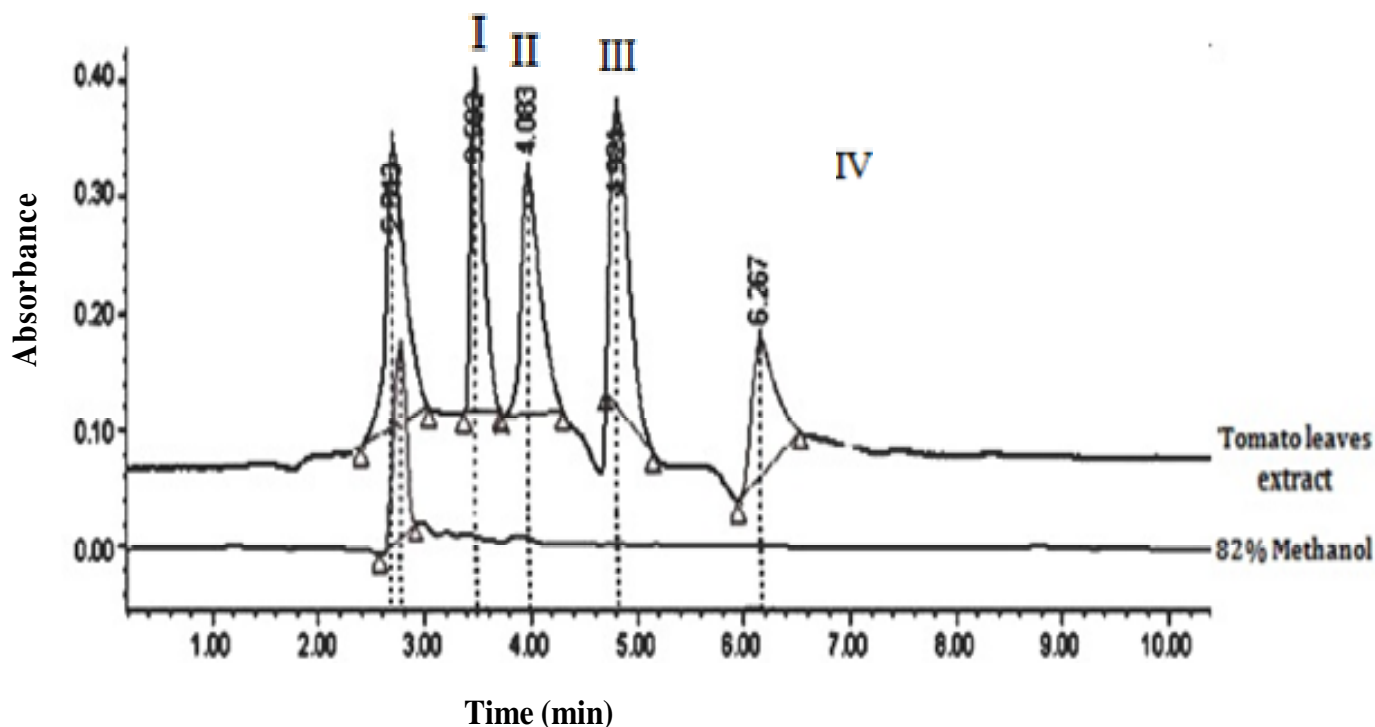
## RESULTS AND DISCUSSION

In HPLC experiment, reversed phase HPLC (RP-HPLC or RPC) was used. The term reversed-phase describes the chromatography mode which is just the opposite of normal phase, namely, the use of a polar mobile phase and a non-polar (hydrophobic) stationary phase (Jeffrey, 2002). The RP-HPLC is considered as the method of choice for the analysis of pharmaceutical compounds for several reasons, such as its compatibility with aqueous and organic solutions as well as its high consistency and repeatability. Sensitivity and accuracy of the RP-HPLC analysis, whether in the pharmaceutical or bioanalytical field, necessitates the use of stationary phases which give symmetrical and efficient peaks.

Furthermore, C18 columns called octadecylsilane (ODS) or RP-18 are currently used in most HPLC methods to separate medicinal materials and mainly the mobile phases used are mixtures of water and acetonitrile (Levin, unpublished).

Short column packed with small particle stationary phase is the column configuration that is most often recommended for use in rapid analysis (www.labbulletin.com, 2009). In isocratic elution, polarity plays an important role in peak resolution. This involves the increase of the interaction between the sample and the stationary phase by increasing the water content, decreasing the flow rate and decreasing the temperature (Kromidas, 2006; Chen and Horváth, 1993). The retention factor increases with decrease of temperature and by simply reducing the amount of organic solvent in the mobile phase will also increase the peak resolution (Sigma, 2009).

Therefore, this purification process has used C18 HPLC column (4.6 × 150 mm, 5 µ) with constant flow rate



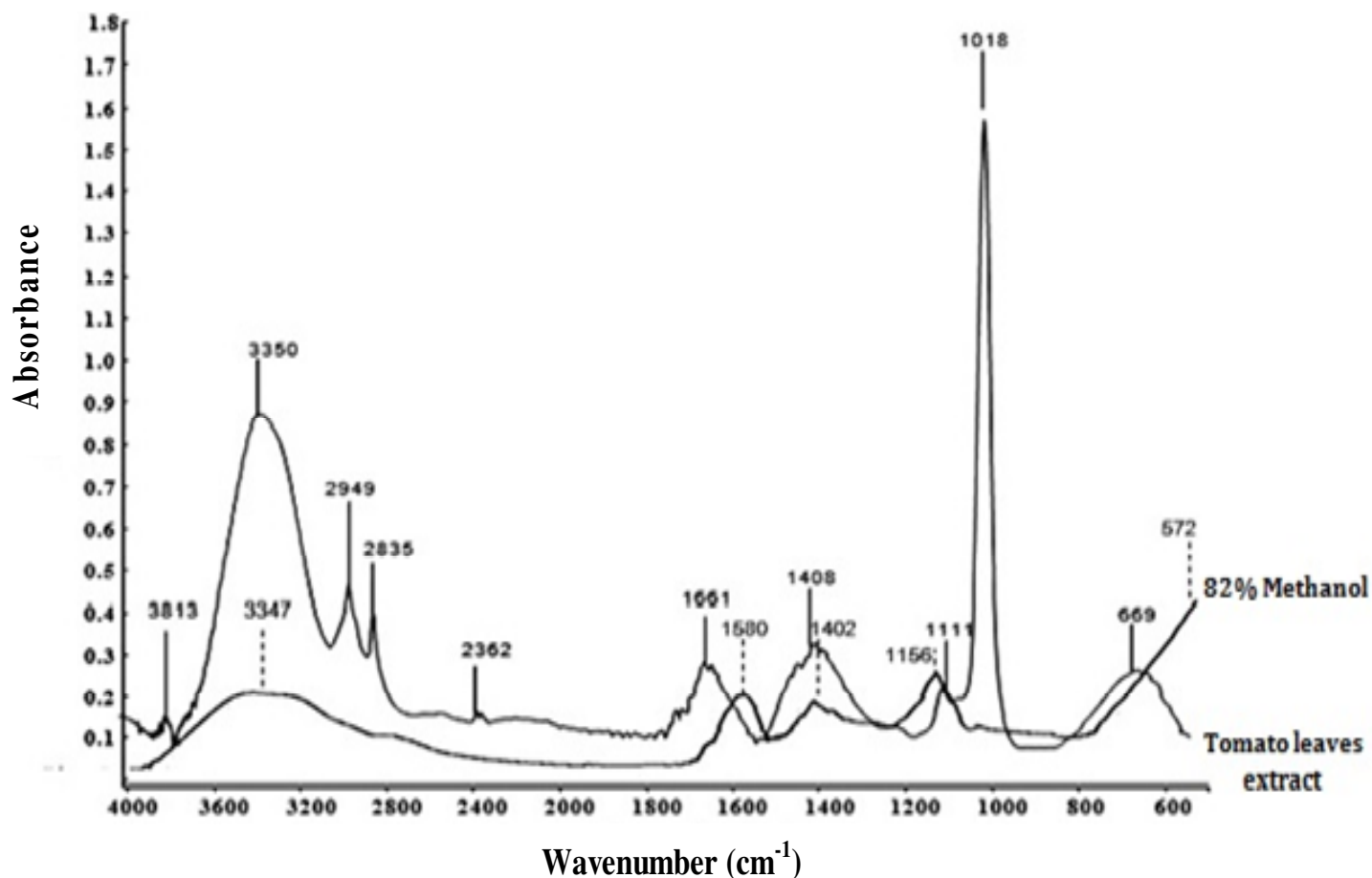
**Figure 1.** HPLC chromatogram of purified tomato leaves extract and 82% methanol (dissolved solvent).

of 1 ml/min, temperature of 30°C and a mobile phase of 30% acetonitrile and 70% water. Cleaning validation tests were performed to assure the cleanliness of the HPLC equipment by flushing water and 100% methanol through the column subsequently before injecting the sample. The strict specifications were applied to track the residues of the analytes, their degradants or impurities and other contaminations left in the column. After allowing the purified sample flow through the column in 30 min, the peaks significantly rose up at retention time of 3.502, 4.083, 4.921 and 6.267 min, which simultaneously represents discernible of four metabolites presents in the active fractions, and neglected the first peak which represents the solvent peak (Figure 1).

According to the column manufacturer datasheet (Ascentis C18, Sigma), four peaks (Figure 1) of uracil, acetophenone, benzene and toluene rose up at I, II, III and IV when using the same condition of this HPLC experiment (Sigma, 2009). Uracil can be used as an anticancer drug that inhibits RNA replication enzymes, thereby eliminating RNA synthesis and stopping the growth of cancerous cells (Garret et al., 1997). In the late 19<sup>th</sup> and early 20<sup>th</sup> centuries, acetophenone was used as a hypnotic and anticonvulsant medicine (Maynard, 1997). It was considered to have greater sedative effects with both paraldehyde and chloral hydrate. On the other hand, presence of benzene and toluene in the plant leaves occurs with the aromatic ring cleavage and their carbon atoms that are mainly incorporated into nonvolatile organic acids, while their incorporation into amino acids is

less intensive (Ugrekheldze et al., 1997). The plant exposure to [1-6(14)C]benzene and [1-(14)C]toluene vapors penetrate into hypostomatous leaves from both sides, whereas hydrocarbons are more intensively absorbed by the stomatiferous side and more actively taken up by young leaves.

However, another research claimed that phenol, dimethylaniline, diethylaniline and di-n-butyl phthalate rose up at I, II, III and IV, respectively (ES, 1998). Phenols also known as phenolic are found in nature, especially in the plant kingdom (Hogan, 2008). In addition, phenolic acids found at high concentrations in a number of plants, possess antioxidant action (Michalak, 2006). The main phenolic acids found are derivatives of 4-hydroxybenzoic and 4-hydroxycinnamic acids. Phenolic acids exert a direct antiproliferative action in human breast cancer cells (Kampa et al., 2004). Dimethylaniline and diethylaniline are the derivatives of aniline which are categorized as aromatic amine (Rai et al., 2004; Poizat et al., 2010). Tomato leaves contain high levels of amines and is an important class of nutraceuticals with strong antioxidant activity and chemotherapeutic effects (Kang et al., 2009). In contrast, different research reported that prednisolone, cortisone and 11 $\alpha$ -hydroxyprogesterone, the derivatives of steroids were observed at I, II and IV, respectively (ES, 2009). Plants from Solanum family have long been the natural supplier of these steroids (Kul'kova et al., 2000). Steroids were found to show significant cytotoxic effects towards human cancer cells (Wu et al., 2009).

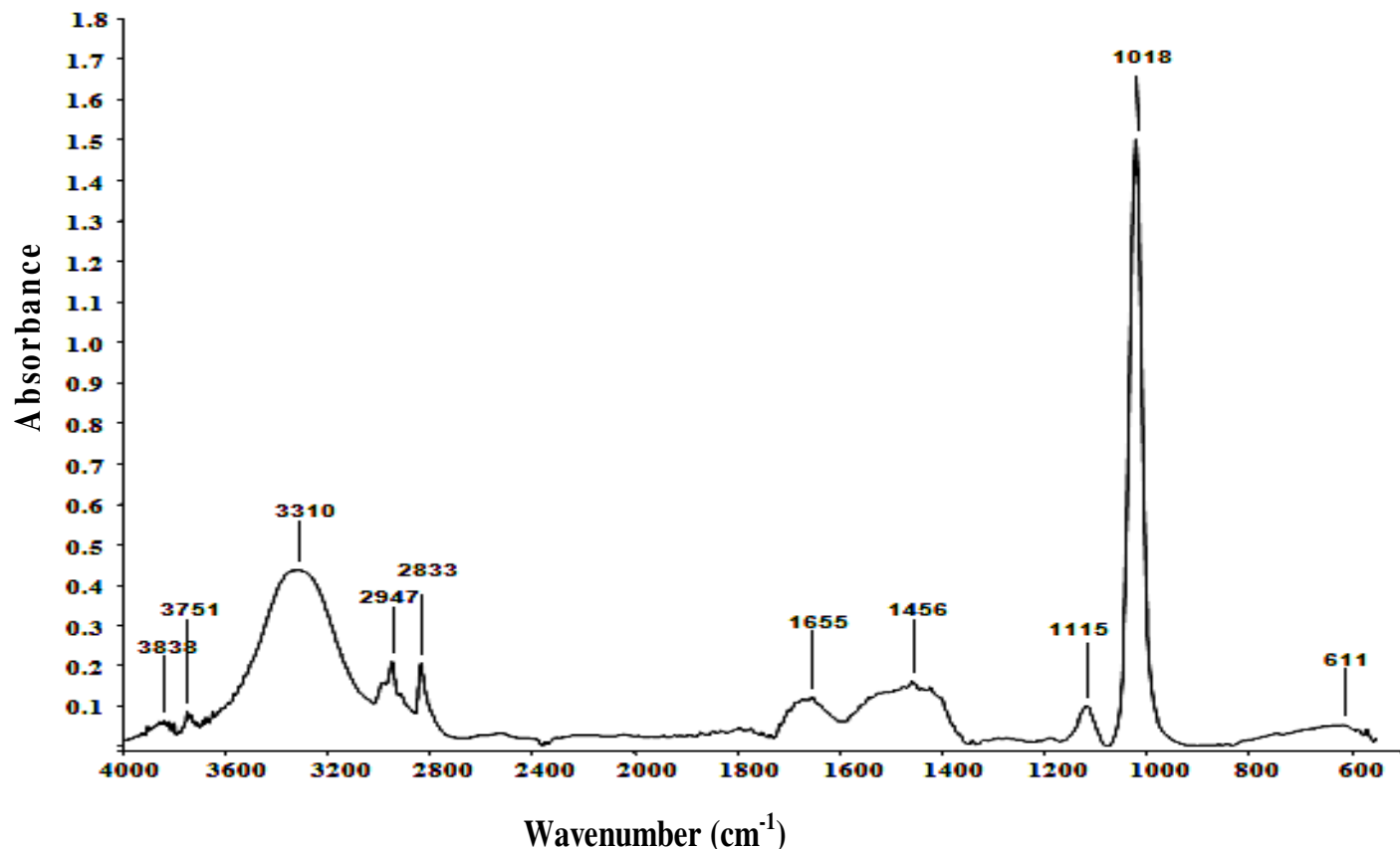


**Figure 2.** An FTIR spectrum of purified tomato leaves extract dissolved in 82% methanol.

Nevertheless, these peaks can be also considered as new unknown compounds that can possibly be influenced by isomerism that depends on their stereochemistry. During the TLC experiment on the active fractions sample, only a single spot appeared on the TLC plate. However, binary verification of the number of compounds present was performed by this HPLC procedure which apparently showed four peaks of metabolites. Isomers are compounds with the same molecular formula but different structural formulas (Smith, 2010). It does not necessarily share similar properties, unless they also have the same functional groups. There are many different classes of isomers such as stereoisomers, enantiomers and geometrical isomers. The main forms of isomerism are structural isomerism and stereoisomerism or spatial isomerism. Molecular isomerism is important in stereochemistry application especially in inorganic chemistry, organic chemistry, physical chemistry and biochemistry. Normally, isomers of a molecule behave alike with identical  $R_f$  value in thin layer chromatography but different retention time in HPLC (Grimme et al., 2007). Even so, further investigation on the presence of compound was performed by FTIR spectroscopy data acquisition.

The use of FTIR spectroscopy is increasing in many fields including drug discovery studies (Gwynne and Heebner, 2010). Figure 2 shows the FTIR spectra of partially purified tomato leaves extract dissolved in 82% methanol and the FTIR spectra of 82% methanol, which operated as a control or a reference. The sample measurement made it possible to eliminate the dissolved solvent influence which is shown in Figure 3. The measurement of the extract may be affected not only by the light-absorption properties of the sample, but also the properties of the reference (Demirdöven et al., 2004). Mathematically, the reference transmission spectrum was subtracted by the sample transmission spectrum. Sample measurement would cancel out the light-absorbing and light-reflecting properties of the 82% methanol. Consequently, the final result only showed the properties of the partially purified tomato leaves extract, approximately. The analytical evaluation of the extract spectrum in terms of functional groups corresponding to absorption of certain frequencies is given in Table 1.

The tabulated data (Table 1) showed the interpretation of the most significant group frequencies for the functional groups and structural components found in the presents compound. The hydroxy (-OH) and amino group



**Figure 3.** A final result of FTIR spectrum of purified tomato leaves extract.

(carbon, hydrogen, oxygen, and nitrogen) are molecular fragment that contribute their own set of characteristic absorptions to the spectrum of the compound. In fact, the bonding between the functional group and the backbone is only one part of the overall picture used for the spectral interpretation (Smith, 1999). The hydroxy groups at frequency 3838 and 3751  $\text{cm}^{-1}$ , may be within the same molecule (intramolecular hydrogen bonding) or they most likely exist between neighboring molecules (intermolecular hydrogen bonding) (Dent and Chalmers, 1997). The impact of the hydrogen bonding is to produce significant band broadening and to lower the mean absorption frequency.

The lowering of the frequency tends to be a function of the degree and strength of the hydrogen bonding (Coates, 2000).

A large shift to lower frequencies was observed at frequency 1655  $\text{cm}^{-1}$ . This amides (carboxylic acids) group can exhibit extremely strong hydrogen bond, forming a stable dimeric structure and highly characteristic (Paul et al., 1997). This absorption is important for the characterization of certain hindered phenol antioxidants (Coates, 1996).

The hydroxyl group simultaneously exhibit alcohol in the extract. Alcohols exist as three distinct classes, which

are primary, secondary and tertiary (Atkins and Carey, 2002). They are distinguished by the degree of carbon substitution on the central hydroxy-substituted carbon, a single substitution being primary, double substitution being secondary, and triple substitution being tertiary. This is an important fact, because the chemistry and oxidation stability of the alcohol are influenced strongly by the degree of substitution (Brown et al., 2000). Whether an alcohol is primary ( $1^\circ$ ), secondary ( $2^\circ$ ) or tertiary ( $3^\circ$ ), may be reflected in the position of the OH stretch absorption, but typically, this is determined by the other absorptions, in particular, the C-O- stretching frequency.

Absorption has less importance, but often characteristically assigned to another form of bending vibration, the out-of-plane bend or wagging vibration of the O-H (Nyquist and Potts, 2001). This can be seen at frequency 611  $\text{cm}^{-1}$ . The OH bending vibrations are broadened by hydrogen bonding as is the stretching absorption, but often to a lesser extent.

In some aspects, the infrared spectra and the characteristic group frequencies of amines tend to be parallel to those of alcohols. The terms primary, secondary and tertiary are used to describe amines, but the substitution relates to the nitrogen, not the adjoining carbon as with alcohols (Kobayashi, 1998). As with hydroxy compounds,

**Table 1.** Functional groups in purified tomato leaves extract spectra.

S/N	Frequency (cm <sup>-1</sup> )	Functional Group	Remark
1	3838 3751	R-OH	Hydroxyl Bonded to saturated carbon
2	3310	R-NH	Aliphatic amines (secondary) Bonded to -NH Symmetric stretching vibration
3	2947	-CH(-CH <sub>2</sub> )	Alkyl group Saturated-CH Asymmetric stretching vibration
4	2833	-R- H <sub>2</sub> C (=O)	Aldehyde group Bonded to unsaturated-CH Symmetric stretching vibration
5	1655	O=CH(-NH <sub>2</sub> )	Amides (carboxylic acid derivatives) Bonded to unsaturated carbon (C=O) stretching vibration
6	1456	-CH(-CH)	Alkyl group Deformation of CH <sub>2</sub> and CH <sub>3</sub> Asymmetric bending vibration
7	1115	C-O-C	Esters Symmetric stretching vibration
8	1018	-CH <sub>2</sub> -NH	Aliphatic amines Bonded to C-N- Asymmetric stretching vibration
9	611	-R-OH	Hydroxyl Bending vibration Weak bands, sometimes absent

hydrogen bonding is important, but the effect tends to be weaker than the hydroxy group, and the overall effect on the spectrum is slightly less pronounced. This situation is altered in the related ammonium and amino salts, where strong hydrogen bonding is experienced and a corresponding broadening of the associated NH absorptions is observed at frequency of 3310 cm<sup>-1</sup> and sharp peak at frequency of 1018 cm<sup>-1</sup>. Note that only the primary and secondary amines exhibit the most characteristic group frequencies, which are associated with the N-H bond (Wishart, unpublished). This peaks present strongly support the previous report that claimed that tomato leaves contains high levels of amines and is an important class of nutraceuticals with strong antioxidant activity and chemotherapeutic effects (Kang et al., 2009). A study by Thornfeldt (unpublished) showed that dermatological

formulation containing 0.1 to 35% weight of a compound selected from the group consisting of aliphatic amines having 9 to 18 carbon atoms together with pharmaceutically acceptable salts is able to treat skin with psoriasis, acne vulgaris, seborrheic dermatitis, atopic eczema or nummular eczema. Thus, suggesting that these diseases can be healed by applying therapeutically effective amount of formulation containing amines to the affected area.

Carbonyl compounds are not only chemically important, but are also important in the interpretation of infrared spectra. The C=O absorption at frequency 2833 cm<sup>-1</sup> is an aldehyde which has the carbonyl group as terminal and only has one substituent, the other being a single hydrogen atom. Conjugation plays an important role in the observed carbonyl frequency (Jose et al., 2003). This

includes connection to an aromatic ring or conjugation to a C=C or another C=O. Lowering of the parent group frequency was observed at frequency  $1115\text{ cm}^{-1}$  which was denoted as ester group. This effect is important for the differentiation of certain types of carbonyl compound, in the determination of whether the carbonyl group is directly or indirectly attached to an aromatic ring (Briggs, 1995). The carbonyl absorption frequency is lowered when the ring is directly conjugated with the ester group. Quinones, for instance, are carbonyl group molecules with a wide range of biological activity (ken et al., 2007). One of their properties is the ability to attract and accumulate electrons in the carbon-hydrogen double bond. Anthraquinones and naphthoquinones are found to play important roles in a wide range of botanical medicines and have been observed to have both anti-tumor and anti-microbial properties (Kenner, 2007).

In addition, the characteristic absorption frequencies also contribute by the parent hydrocarbon species and the associated backbone or substituent group, which includes aliphatic structures such as alkyl group (Yeh and Frederick, 2010). The spectral contributions are characterized as C-H stretching at frequency 2947 and  $1456\text{ cm}^{-1}$ , which for the most part, are unique for each molecule, and generally described as skeletal vibrations. The C-H stretch vibrations for strong methyl bands, showing significant splitting band indicate chain branching.

The saturated hydrocarbon C-H stretching absorptions all occur below  $3000\text{ cm}^{-1}$  (MSU, 2010).

In this study, the data for an infrared spectrum were shown based on the most common and characteristic group frequencies.

The present data analysis helps in understanding of the chemical functionality of the compound in the sample. Nuclear magnetic resonance (NMR) can give more information about the compound in the sample in future research. However, this basic interpretation was accomplished and the sample was partially characterized. Nevertheless, the sample could be a mixture of compounds since it was a partially purified sample. In such a case, an exact match may not be possible, unless the reference spectrum exists for the formulation.

## Conclusion

HPLC as well as FTIR spectroscopy can be used to analyze the presence of anticancer component in the partially purified tomato leaves extract. Expectations should focus on the fields of metabolites prediction by HPLC, which will permit higher level analysis with enhanced analytic capabilities and reduced analyzing time.

When combined with infrared spectroscopy, the result of a positive identification of qualitative analysis in different kinds of materials can be accomplished. Therefore, this research had significantly identified the properties of compound present in the tomato leaves, which are asso-

ciated with cancer treatment.

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