# Marker to authenticate *Eurycoma longifolia* (Tongkat Ali) containing aphrodisiac herbal products

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**The benefits of *Eurycoma longifolia* (Tongkat Ali, TA) for its aphrodisiac capabilities are well known and many products are marketed worldwide. Due to its popularity, the plant is being abused for promoting fake products. Therefore, there is a need for better testing of the markers required by authorities and responsible manufacturers. A low-molecular-weight protein has been studied for developing it as a testing marker. Two dimensional electrophoresis (2DE) (four spots were observed) was used for positive detection of proteins in an aqueous extract of TA root and the pronounced separation of a Coomassie-stained spot, subsequently referred to as Marker A. Consecutive chromatographic separations of the aqueous extract of TA led to the isolation of pure protein from Marker A. When this marker was used to test 46 TA-based products randomly selected from markets worldwide, in 20 of them, the results were found to be comparable to those obtained using the organic eurycomanone marker. The ranking of products from the highest quantity to the lowest observed to be ordered differently if compared for both markers. This is an expected outcome because Marker A was measured for its protein content and eurycomanone for its organic molecule. Marker A detection using 2DE is shown to be a useful tool to test products supplemented with *E. longifolia* root.**

**Keywords:** Authentication, electrophoresis, *Eurycoma longifolia*, herbal products, markers.

HERBAL plants have been trusted for generations by indigenous people and remain important in contemporary times, providing healing to many around the world. Some plants are popular worldwide and others are popular within the confines of their country of origin. *Eurycoma longifolia* or Tongkat Ali (TA) is a plant that bridges boundaries, and a search using keywords 'Tongkat Ali' on Amazon.com, generates thousands of hits. In addition to the most notorious use of TA as an aphrodisiac, traditional uses of this plant range from post-natal tonic to the treatment of malaria. Published studies have identified quassinoids, alkaloids, squalene derivatives, tirucallane-type triterpenes and biphenylneolignans of this plant that have the potential to treat various diseases, such as malaria and cancer[1].

Currently, there are numerous TA-based products in the market due to the energy-enhancing and aphrodisiac properties of this plant[2]. These products range from capsules, tablets, liquid formulations, premixed coffee and canned processed drinks[3]. The standardized water-soluble extract was patented in 2006 (US 7.132.117.B2) after having undergone extensive animal and human clinical evaluation[4]. Usually, the concentration of TA in commercially available products is reported as $x : y$, where $x$ grams of TA is used to produce $y$ grams of extract. However, this claim is insufficient for authentication purposes. Taking advantage of the lucrative market for this plant, some manufacturers are suspected to deceive consumers by adding very little of the TA root or the less potent bark of the tree, leading to lower doses than required for therapeutic benefits. At times, substances that are not declared on the packaging, such as controlled erectile dysfunction drugs in cheap generic forms or other less-capable aphrodisiac plants are also added, placing consumers at risk of using fake products. Due to these challenges, rigorous quality control of TA products is warranted. Herbal medicines in the market are usually assessed only for safety; however, there needs to be a quality paradigm that will address both safety and efficacy[5].

To date, there is no method to authenticate TA-based products, except by determining the presence and concentration of the major compound in the plant, i.e. eurycomanone, using HPLC[6]. It is unclear which category this marker falls under because, until now, there has been only one publication, by Low *et al.*[7], that showed the efficacy of eurycomanone towards the aphrodisiac property of this plant, and there remains ambiguity regarding the contribution of eurycomanone to the aphrodisiac property of this plant.

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In contrast, some evidence has suggested that a likely active constituent for the aphrodisiac capabilities is a protein. A patent stated that the entity contributing to the property of sexual enhancement could be a peptide[8]. Vejayan *et al.*[9] detected two distinct protein markers by performing two-dimensional electrophoresis (2DE) of standardized extracts and of several TA-based products sold in Malaysia. The marker was not detected in selected herbal products devoid of TA extracts. Further exploration of this durable, heat-stable protein or peptide as another marker to authenticate TA-based products is carried out in the present study.

## Material and methods

### *Aqueous extraction of TA roots*

Fifty grams of *E. longifolia* root powder was added to 600 ml of deionized water. The sample then was boiled under reflux for 5 h followed by filtration with Whatman No. 1 filter paper and freeze-drying[6].

### *SDS-PAGE*

SDS-PAGE was performed according to the method described by Laemmli and Favre[10], with modifications. Electrophoresis was carried out on a 15% resolving gel for approximately 1½ h using a mini vertical slab gel system (BioRad Laboratories, USA). A total of 2.5 mg of sample was dissolved in 200 µl of rehydration buffer and allowed to stand for 15 min before pipetting 10 µl of this preparation into a well within the stacking gel. The protein was stained using Coomassie brilliant blue solution (PhastGel™ Blue R; GE Healthcare, USA) and destained appropriately in 10% acetic acid preparations.

### *Linear-mode MALDI analysis*

MALDI TOF MS analysis was performed using a AXIMA-CFR instrument (Shimadzu-Kratos Corp., Japan) operating in positive ion linear mode in a mass range of $m/z$ 200–2000 with a resolving power of $10^6$ and laser energy of 15 µJ per laser shot. A total of 1.5% (w/v) of each TA sample was dissolved in ultra-pure water, and 5 µl of this preparation was mixed with an equal volume of ACH-cinnamic acid (Amersham Biosciences AB, Sweden) and spotted onto a MALDI sample slide.

### *Purification of protein from E. longifolia crude extract*

*E. longifolia* crude extract was pre-fractionated by size-exclusion chromatography using an AKTA protein purification system (GE Healthcare) by measuring absorbance at 280 nm and using a 20 cm × 3 cm column containing Sephadex G25 Superfine resin (GE Healthcare). Elution was carried out at a flow rate of 2 ml/min for five column volumes, and the fraction collector was programmed to collect 2 ml per fraction. Further purification of the fraction of interest was completed with Q Sepharose High Performance beads (GE Healthcare) packed into an XK16 column; a flow rate of 1.5 ml/min was used. The column was equilibrated with three column volumes of buffer (20 mM *Tris*-HCl, pH 8.7). Next, elution was carried out in stepwise gradient mode, starting with 60% elution buffer (0.5 M NaCl in 20 mM *Tris*-HCl, pH 8.7) followed by 65% elution buffer and ending with 70% and 90% elution buffer, using one column volume for each; 2 ml fractions were collected. A 5 ml HiTrap desalting column (GE Healthcare) was used for desalting. All fractions were freeze-dried appropriately.

### *Product selection and acquisition*

To analyse the quality of TA-based products using organic eurycomanone and protein markers, various commercially available TA-based products were sampled. Samples were collected from multiple sources, such as pharmacies, night markets, jamu shops, food courts and even on-line stores. A total of 46 selected products claiming to contain TA in various pharmaceutical dosage forms, either registered or unregistered with the National Pharmaceutical Control Bureau (NPCB) of Malaysia, were sourced from Malaysia (the natural habitat for this plant) as well as from international markets. The products were labelled as either *E. longifolia* or Tongkat Ali. To make the sampling as random as possible, samples were chosen based on recommendations by vendors at the selected premises. The products were assigned codes in the order of sampling (C1, C2, C3, etc.).

### *HPLC analysis of products*

An HPLC instrument (Waters, USA) with a photodiode array detector and an XBridge column (Supelcosil C18 5 µm, 250 mm × 4.6 mm) was used. The mobile phase consisted of an isocratic mixture of water and acetonitrile (Fisher Scientific, UK) (86 : 14, v:v) at a flow rate of 0.8 ml/min, and absorbance was measured at 238 nm. A standard stock solution (2.5 mg/ml) of eurycomanone (ChromaDex, USA) was prepared. A calibration curve was generated by injecting serial dilutions of this standard solutions into the HPLC instrument. For each product, 5 mg of the contents of a capsule, drink sachet, tablet or other similar preparations was dissolved in 1.5 ml of water and filtered through a 0.45 µm membrane filter before being injected into the HPLC instrument. The eurycomanone content of each product was calculated as w/v%.

### *Two dimensional electrophoresis of products*

A 2DE analysis of the protein samples in this study was performed as previously described[9] with a few modifications. Briefly, a 7 cm long IPG strip was used for isoelectric focusing (IEF), while a homogenous 15% gel was used for SDS-PAGE. Gels were stained with 0.25% (w/v) PhastGel™ Blue R (GE Healthcare) in 1 : 4 (v/v) acetic acid : water and destained with several changes of 16% (v/v) acetic acid until a colourless background was obtained. Gels were analysed using ImageMaster™ 2D Platinum 7.0 software (GE Healthcare). Differences between corresponding spots were analysed in each set of gels. A gel that was run using purified protein was used as reference.

### *De novo sequencing*

*De novo* sequencing analysis was carried out as a service provided by the Australian Proteome Analysis Facility (APAF), Australia. Briefly, the method used was as follows: protein spots from 2DE gels were excised appropriately and digested with trypsin. The gel samples were destained with ammonium bicarbonate/acetonitrile and subjected to a 16 h tryptic digest at 37°C in 25 mM ammonium bicarbonate (160 µl). The supernatant from the digest was extracted for analysis. The sample (40 µl) was injected onto a peptide trap (Michrom Bioresources, Inc, CA, USA) for pre-concentration and desalted with 0.1% formic acid and 2% acetonitrile at 8 µl/min. The peptide trap was then placed in line with the analytical column. Peptides were eluted from the column (SGE ProteCol C18, 300A, 3 µm, 150 µm × 10 cm) using a linear solvent gradient with steps from $H_2O$:$CH_3CN$ (90 : 10; + 0.1% formic acid) to $H_2O$:$CH_3CN$ (0 : 100 + 0.1% formic acid) at 500 nl/min over a 50 min period. The LC eluent was subjected to positive ion nanoflow electrospray MS analysis on a QStar Elite system (AB Sciex).

## Results

### *Gel electrophoresis and linear mode MALDI-TOF MS*

Initially, protein content of the crude extract of *E. longifolia* root was characterized using general protein analysis techniques. As shown in Figure 1 *a*, only one protein band was observed by analytical SDS-PAGE of the TA extract, with an approximate molecular weight of 10 kDa based on a calibration curve of the log molecular weight of the protein standard versus migration distance. By 2DE, we were able to separate proteins based on isoelectric point in addition to molecular weight. As shown in Figure 1 *b*, the completed 2DE of the crude extract had four spots, with spots A and B being more intense than the others in the same low-molecular-weight region, but with different isoelectric points. Consequently, linear mode MALDI TOF MS analysis of the crude extract exhibited a signature 4.3 kDa peak. Figure 1 *c* shows the mass spectra of the crude plant extract supplemented with maltodextrin and cyclodextrin excipients; the mass spectra of all three samples exhibits similar 4.3 kDa peaks.

### *Sequential chromatography to purify marker A*

Two-step protein chromatography, involving an initial size-exclusion step (to eliminate non-protein micro-organic molecules) followed by ion-exchange chromatography (to separate the protein by charge), yielded a single protein spot in the 2DE gel (Figure 2). Hence, the outcome of stepwise chromatography decisively eliminated the proteins in spots B, C and D (Figure 1 *b*).

### *De novo sequencing of marker A*

Table 1 shows the peptide masses obtained from the tryptic digest of the 2DE gel spot corresponding to Marker A. A BLAST search conducted on these ten peptides was unable to significantly match these peptides to any protein in the protein database due to novelty of the proteins in *E. longifolia*.

### *Comparison of eurycomanone and marker A*

After isolation of marker A, we compared the protein marker to the organic marker, eurycomanone. Quantification of Marker A in the samples was performed using the 2DE software ImageMaster 2D Platinum 7 (IMP7). The gels were first viewed three-dimensionally, in which each protein spot was converted to a peak; the height of the peak indicated the intensity of the protein. Figure 3 *a–c* shows an example of a three-dimensional view obtained from the software. Figure 3 *d* and *e* respectively, show the profile of Marker A and eurycomanone in a product known to contain multiple formulations.

To quantify Marker A in all samples, the spot corresponding to it in the 2DE gel of each product was normalized to that in the gel of purified Marker A (Figure 2 *c*) using ImageMaster 2D Platinum 7 software. The amount was quantified based on fold differences in the level of Marker A. The eurycomanone content of each product was calculated by determining the amount injected by extrapolating from a calibration curve of standard eurycomanone followed by calculating %w/v (ref. 6). Table 2 lists the eurycomanone and protein marker contents of the tested samples.

Analysis of the 46 randomly selected TA-based products for both the organic and protein markers revealed that both markers were present in 20 of the 46 products. Interestingly, the remaining products, constituting more than 50% of the total, were found to be devoid of either markers, hence disputing the authenticity of these

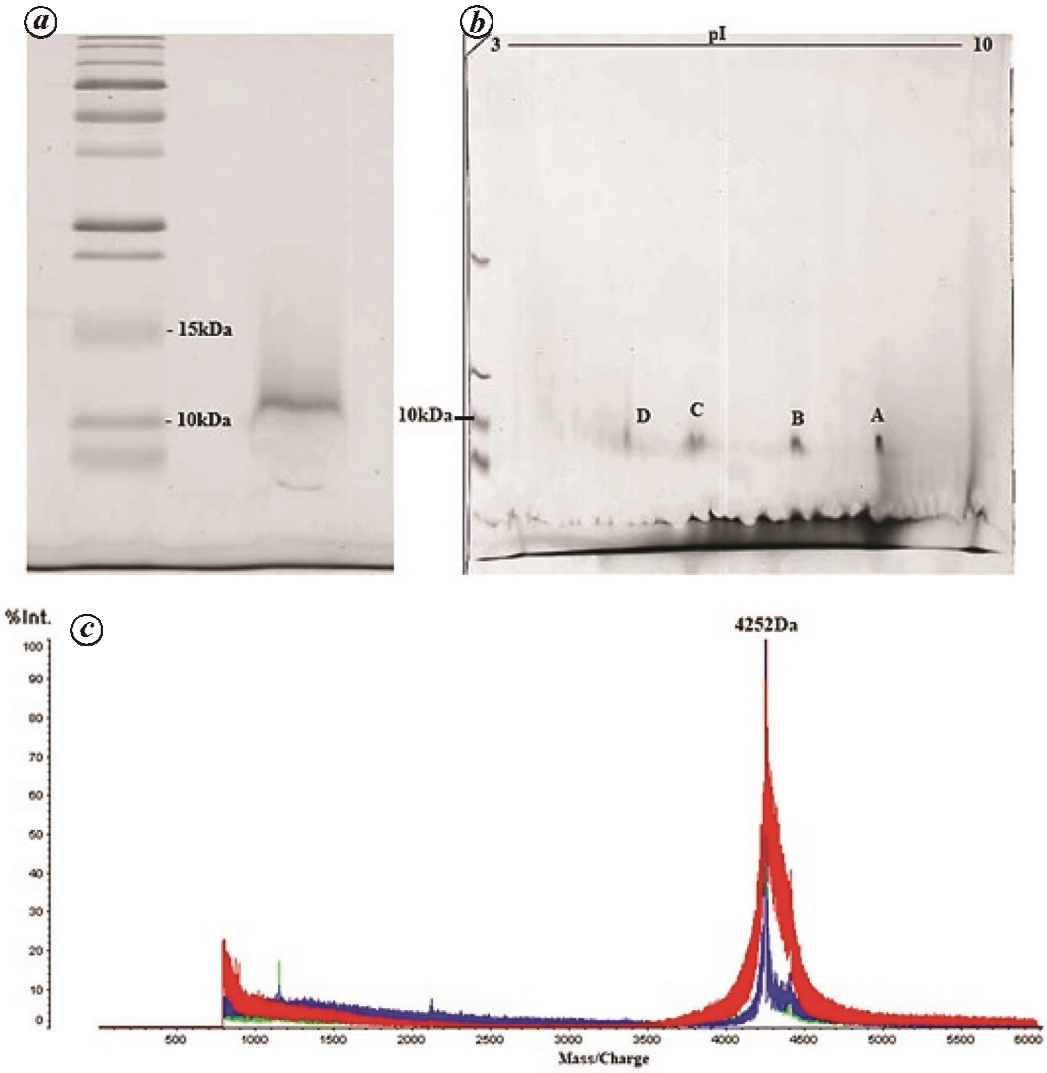


**Figure 1.** ***a***, SDS-PAGE of crude extract (2.5 µg loaded) using a homogenous 15% gel. ***b***, 2DE gel of crude extract (2.5 µg loaded) with IEF on a 7-cm IPG strip, both stained with Coomassie blue. ***c***, superimposed linear mode MALDI-TOF MS spectra of ( ) crude extract, ( ) crude extract + maltodextrin and ( ) crude extract + cyclodextrin.

**Table 1.** Fragmentation list for marker A, as determined by APAF

| Peptide mass after ESI-MS/MS run | Most significant amino acid sequences predicted | BLAST search on NCBI |
|---|---|---|
| 467.3 | LLGMDGGVR | No significant match |
| 530.8 | TLLDDAGLDK | |
| 590.3 | YEELGALTAGR | |
| 613.6 | YDCPNGGALASGFGAAVAK | |
| 627.8 | DMSGAGGAGMAVAK | |
| 711.3 | TQGASMYGMTLMGYGPGYAK | |
| 742.4 | TSAAHHVTEGEGGGEGAMLGGAGR | |
| 744.4 | NGTLGATEVGSTGAPR | |
| 1196.5 | QPAYVSSDLDSNGPLAGGMGAAVAK | |
| 1204.5 | QGPGGSSGGYEVCLPTAGGDSGSVAK | |

products and the claim that they contain TA as labelled. The crude TA root extract was found to have the highest levels of both markers and the negative control (capsules of another aphrodisiac plant, 'Ubi Jaga' or *Smilax myosotifolia*) yielded negative results. Both markers were not useful in the detection of other types of TA (crude

extract TA1 and TA2 in Table 2), i.e. those not belonging to *E. longifolia*. Though the markers exhibited similar trends when detected, the amount of each marker detected differed between products, e.g. although product C28 had the highest levels of the eurycomanone organic marker, it was only the eighth highest with respect to the levels of protein marker A. All the beverage-based products, i.e. D1–D8 did not exhibit the presence of markers.

## Discussion

All three characterization techniques, i.e. SDS-PAGE, 2DE and linear mode MALDI enabled the detection of proteins in TA extract or TA-based products. Previous works have established the use of SELDI-MS (somewhat similar to MALDI-MS) and 2DE for the analysis of TA extracts and related products[6,11]. Analysis of TA supplemented with maltodextrin and cyclodextrin provided a 4.3 kDa signature peak in linear-mode MALDI spectra. Both these excipients are commonly used in herbal preparations and drugs[12–14]. The 2DE gels could only be stained with Coomasie stain to reveal marker A. It is likely that the proteins in *E. longifolia* are glycosylated, as silver staining is known to be less sensitive for the detection of highly glycosylated proteins[15,16].

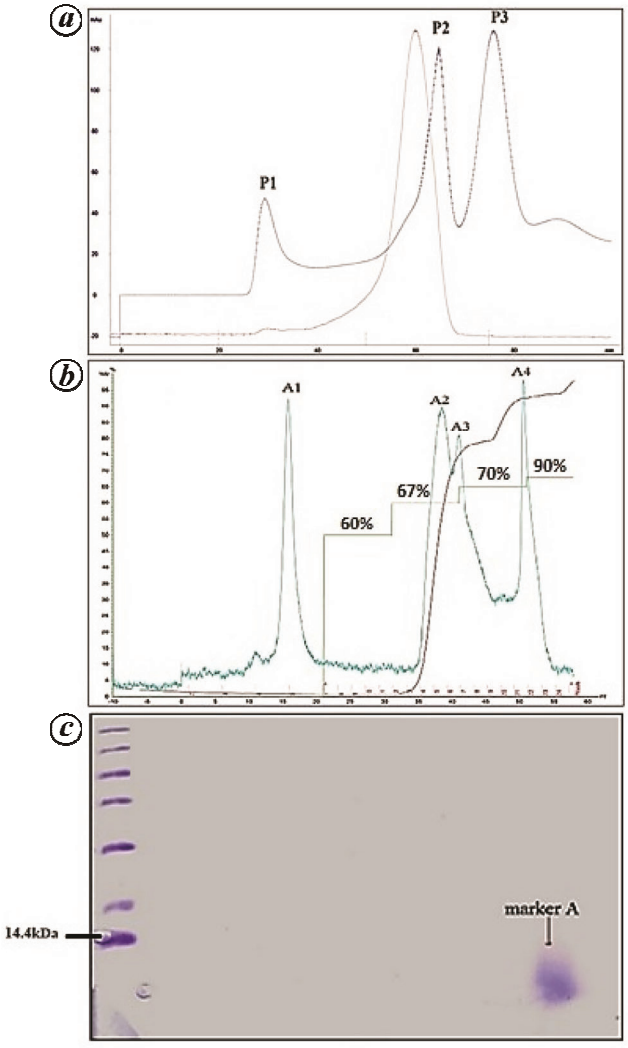


**Figure 2.** ***a***, Chromatogram of aqueous extract of Tongkat Ali roots, obtained by size-exclusion chromatography, showing three peaks (P1–P3). ***b***, Elution profile of P1 obtained by anion-exchange chromatography, with 0.5 M *Tris*-HCl (pH 8.7) as binding buffer and 0.5 mM NaCl in 0.5 M Tris-HCl (pH 8.7) as elution buffer. Four peaks (A1–A4) can be observed. Green line indicates concentration of the elution buffer, while brown line indicates conductivity. ***c***, 2DE profile of fraction A4, exhibiting a single spot.

The isolation of Marker A from the crude extract of TA is required beforehand in order to determine the corresponding amounts of this marker in the 2DE profiles of TA-based products. This can be achieved easily by two-step sequential chromatography; however, identification of protein in Marker A was unsuccessful due to the novelty of the protein, especially given that the database had only 12 proteins, none of which was significantly related to the marker[17]. Although alternative techniques, such as the tedious and expensive N-terminal sequencing, are available to determine complete protein sequences, such need did not arise in this study.

Both markers were useful for the detection of compounds present in TA extracts to determine the authenticity of products containing this herbal plant; however, the outcome was not positive for TA-based beverages, e.g. premixed coffee tested. TA-based beverages are known to largely contain coffee with a very low amount of TA added, for cost-saving purposes, which explains why neither marker was detected in the beverage products tested in this study. Overall, consumers should be wary of the benefits of such products, even though a study of 200 TA-coffee drinkers indicated positive product attributes such as customer preference, positive customer experience, customer satisfaction and lasting customer loyalty[18].

The term 'Tongkat Ali' is used regionally in Malaysia to describe three types of plants, i.e. Tongkat Ali Putih (*E. longifolia*), Tongkat Ali Merah (*Stema tuberosa*) and 'Tongkat Ali Hitam' (*Polyalthia bullata*)[19]. 'Putih', 'Merah' and 'Hitam' can be translated from Malay as white, red and black respectively, describing the colour on the outer section of the root. In the present study, both markers were only useful to authenticate 'Tongkat Ali Putih'. This was expected, as the other two plants belong to an entirely different genus and species. Nevertheless, it was impossible to ascertain which of the three types of the plants had been used with certainty by the manufacturers in their products, as the labels only identified 'Tongkat Ali'.

It has been established that the amount of the markers detected varies among the products, which have been ranked in descending order of the amount of eurycomanone marker. This result is clearly due to the involvement of two different constituents of TA, organic eurycomanone

**Table 2.** Marker content in the samples tested

| Sample | Herbal content (as stated in the packaging label) | Eurycomanone content (% w/v) | Marker A (µg) |
|---|---|---|---|
| Crude extracts (positive control) | Freeze dried *Eurycoma longifolia* | 15.15 | 13.3 |
| C28 | *E. longifolia*, *S. asiatica*, *A. sativum*, *A. angullia*, *Z. officinale* and *S. myositiflora* | 3.82 | 5.7 |
| C29 | Tongkat Ali | 3.24 | 8.2 |
| C11 | Tongkat Ali and Ginseng | 2.95 | 5.1 |
| C2 | *E. longifolia* | 2.27 | 4.3 |
| C41 | *E. longifolia* | 2.12 | 10.1 |
| C19 | Tongkat Ali | 1.96 | 11.7 |
| C14 | *E. longifolia* | 1.72 | 8.3 |
| C27 | *E. longifolia*, *T. terrestris*, *P. nigrum*, *W. somnifera*, *E. aromatic* and *C. asiatica* | 1.66 | 8.5 |
| C26 | *E. longifolia*, *G. flubus*, *S. myostiflora*, *S. artaboltys*, *F. acuminata*, *H. zeylanica*, *G. panicoides* and *G. pendula* | 1.03 | 2.4 |
| C16 | Tongkat Ali and Ubi Jaga | 0.99 | 7.9 |
| C40 | *E. longifolia* | 0.84 | 10.3 |
| C6 | *E. longifolia*, *Artaboltrys* sp., *S. myostiflora*, *P. neriifolius*, *H. zeylanica* and *A. indica* | 0.75 | 8.2 |
| C34 | *E. longifolia* | 0.55 | 10.5 |
| C18 | Tongkat Ali, Ranjang Besi, Ranjang Tembaga, Gajah Beranak, Akar Pawang and Akar Gamat | 0.40 | 9.3 |
| C24 | *E. longifolia*, *S. myosotiflora*, *N. sativa*, *A. malacensis*, *T. foenum graecum*, *Z. minus*, *L. flavenscens*, *A. calamus*, *C. sativum*, *P. longum* and *T. ammi* | 0.36 | 2.6 |
| C31 | Tongkat Ali, Ubi Jaga and Tongkat Ali Hitam | 0.31 | 5.6 |
| C17 | Tongkat Ali, Ubi Jaga, Ranjang Besi, Ranjang Tembaga and Gajah Beranak | 0.26 | 6.1 |
| C15 | Tongkat Ali, Ubi Jaga, Ranjang Besi and Akar Pawang | 0.22 | 4.3 |
| C38 | *E. longifolia* | 0.19 | 7.2 |
| C23 | *E. longifolia*, *P. anisum*, *C. cyminum*, *Z. officinale*, *C. sativum*, *A. galanga*, *C. zadoaria*, Garlic, *S. variegatus* and *P. nigrum* | 0.16 | 2.9 |
| C8 | *E. longifolia*, maca, ginger, rice bran and black seed | Nd | Nd |
| C9 | *E. longifolia*, *E. brevicornum*, *Actinolitum*, *C. songaricum*, *C. deserticola*, *C. chinensis* and *C. Deserticola* | Nd | Nd |
| C20 | *E. longifolia*, *N. sativa*, *F. vulgare, G. pendula*, *C. domestica, N. fructus* and *L. galangal* | Nd | Nd |
| C25 | *E. longifolia*, *F. malaccensis* and *C. xanthorriza* | Nd | Nd |
| C3 | *E. longifolia*, *S. myostiflora*, *T. crispa*, *F. callicarpa*, *N. sativa*, *Z. officinale* and *P. cubeba* | Nd | Nd |
| C35 | *E. longifolia*, *C. cyminum*, *N. sativa*, *S. myostiflore*, *A. calamus* and *P. anisum* | Nd | Nd |
| C10 | *E. longifolia*, Ganoderma, *H. pantrocinum*, *Yohimbea*, *P. ginseng*, *S. biaculeatus* and *Pantrocinum* | Nd | Nd |
| C22 | *E. longifolia*, *Z. officinale*, *E. aromatica*, *A. calamus*, *N. sativa*, *C. sativum*, *P. longum*, *T. ammi*, *P. anisum* and *C. caudatum* | Nd | Nd |
| C30 | *E. longifolia*, *M. fragrans*, *Zingiber* sp. and *Curcumae*, Yohimbin | Nd | Nd |
| C5 | *E. longifolia* | Nd | Nd |
| C12 | *E. longifolia* | Nd | Nd |
| C13 | Tongkat Ali, maca, Ginseng (Siberian/Korean) | Nd | Nd |
| C32 | Tongkat Ali, horney goat weed, saw palmetto, orchic substance, wild yam, sarsaparilla and nettle root | Nd | Nd |
| C33 | Tongkat Ali, sarsaparilla, pumpkin powder, *M. puama*, oat straw, nettle, cayenne paper, astragalus, catauba, licorice, *T. terrestris* and orchic oyster | Nd | Nd |
| C39 | Tongkat Ali and Panax Ginseng, Horney goat weed, maca, *X. scabrosa*, *C. monieri*, *M. pruriens*, guarana, wild yam and maca | Nd | Nd |
| C36 | *E. longifolia* | Nd | Nd |
| C37 | Tongkat Ali, maca, Ginseng (Siberian/Korean) | Nd | Nd |
| C42 | *E. longifolia*, *A. sinensis*, *P. roxburghil*, *C. xantthorria*, honey, *B. carteri*, *N. sativa*, *P. nigrum*, *C. cyminum* and *C. pillosula* | Nd | Nd |
| D1 | Ginseng and Tongkat Ali | Nd | Nd |
| D2 | Tongkat Ali, Ubi Jaga and Ganoderma | Nd | Nd |
| D3 | Tongkat Ali | Nd | Nd |
| D4 | Tongkat Ali and Mahkota Dewa | Nd | Nd |
| D5 | Tongkat Ali and Ginseng | Nd | Nd |
| D6 | Tongkat Ali and cordiseps | Nd | Nd |
| D7 | Tongkat Ali, Ubi Jaga, Tongkat Samad and Stevia | Nd | Nd |
| D8 | Tongkat Ali, Guarana and Maca | Nd | Nd |
| Negative control | 'Ubi Jaga' or *Smilax myosotifolia* | Nd | Nd |
| Crude extract TA1 | Tongkat Ali Merah, 'Akar Haji Samad' or *Stema tuberosa* | Nd | Nd |
| Crude extract TA2 | Tongkat Ali Hitam, 'Pokok Peleh Angin' or *Polyalthia bullata* | Nd | Nd |

Nd, Not detected. Samples are arranged in the order of eurycomanone level, from highest to lowest.

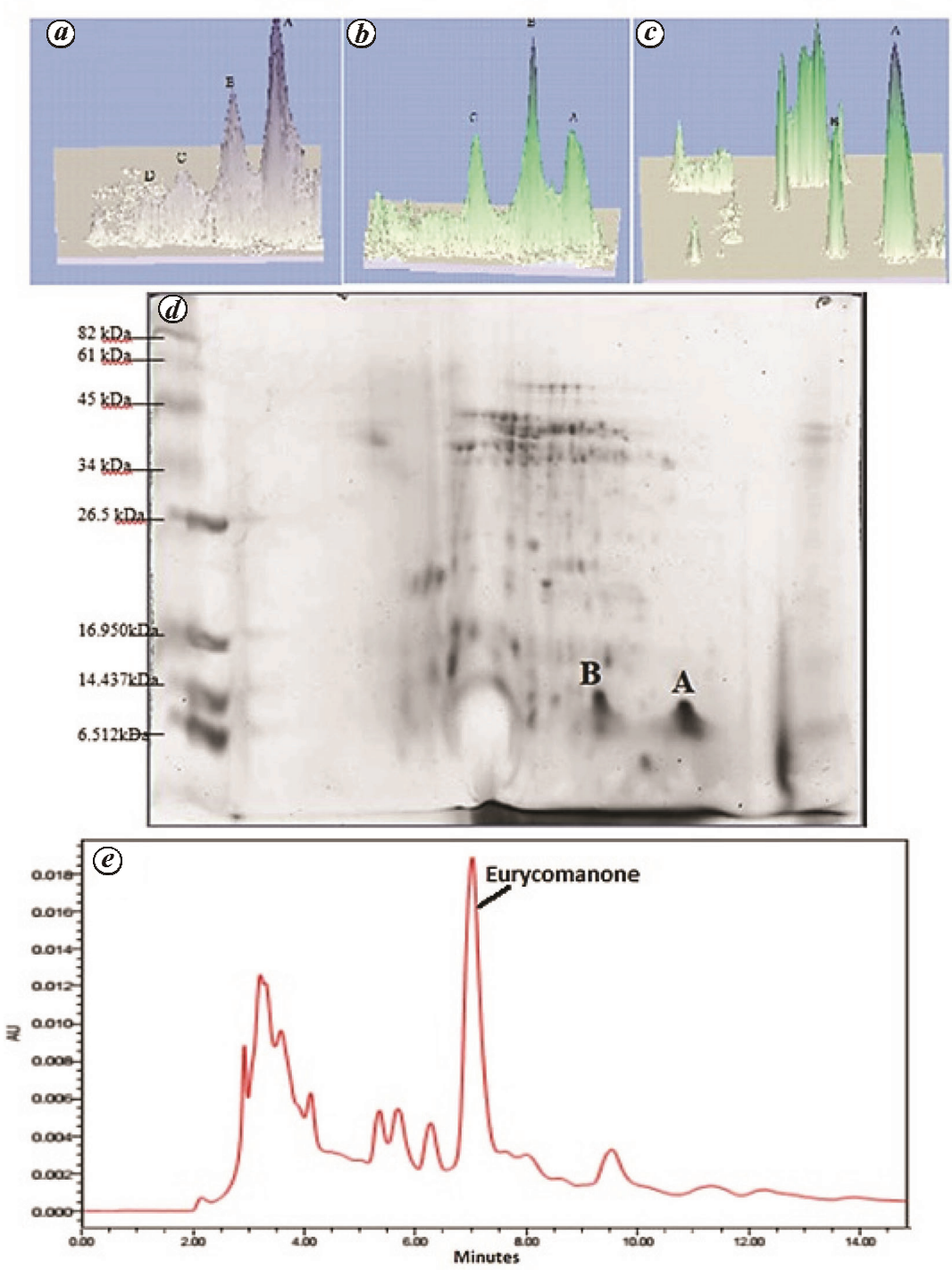


**Figure 3.** Three-dimensional view obtained using ImageMaster2D Platinum 7 software. The height of the peak indicates intensity of the corresponding spot. ***a***, Freeze-dried *Eurycoma longifolia* extract. ***b***, Sample with single formulation (C2). ***c***, Sample with multiple formulations (C24). ***d***, ***e***, Actual 2DE image and HPLC profile respectively, of a sample with multiple formulations (C24).

and a protein. The advantage of the latter as a useful TA marker is that this marker can be utilized to generate an antibody, potentially resulting in a portable and useful biosensor to deter unscrupulous TA exporters and product manufacturers. An array of studies on cost-effective, rapid and high-throughput quality control to determine adulteration and authenticity of natural products is gaining interest[20].

Unfortunately, in an attempt to establish this new protein marker, it was found that 50% or more of the products tested negative for both markers even though the product labels clearly stated that the products contained

'Tongkat Ali' or '*Eurycoma longifolia*'. The herbal industry is a large multibillion-dollar market[21–23]. This lucrative market has been the target of certain unethical manufacturers of herbal products, who are willing to exploit the popularity of herbal or spice plants to benefit their sales. In the past, the popularity of TA has been exploited, and numerous arrests have been made for the sale of fake products[24]. It has been suspected that some TA-based products are not entirely authentic, and instead of the more potent root, the stem, which is difficult to differentiate, has been used. Another method of manipulation in many countries is the addition of cheap, synthetic, generic phosphodiesterase type-5 enzyme inhibitors, e.g. sildenafil citrate or tadalafil to herbal products that claim to be natural products for the treatment of erectile dysfunction[25–27].

Both markers for their utilization in routine quantitative authentication require pure isolates for eurycomanone and protein of Marker A. Eurycomanone can be purchased easily from many reputable suppliers, though priced exorbitantly for a few milligrams. Some workers have managed to reduce the extraction time to less than 20 min compared to 16 h using the conventional distillation technique[28]. For the marker A protein, the challenge resides not with its purification but the tedious 2DE procedures. This technique of proteomics is recently experiencing facing-out in Western laboratories and may soon become the same situation worldwide. Although it is expected that utilization of 2DE modified versions (e.g. 2DE-DIGE) may save it from total extinction altogether[29]. Hence for better use of Marker A, the more popular non-gel based proteomics, i.e. liquid chromatography mass spectrometry (LCMS) is recommended. LCMS allows a new database to be derived for Marker A protein and therefore eliminates the necessity to purify this protein often, as is the requirement for 2DE. LCMS favours low-molecular-weight proteins and is much faster in running and analysis compared to 2DE.

## Conclusion

The organic eurycomanone marker is useful for quality control and quality assurance of TA-based herbal products. Regulatory bodies have utilized this marker to test the authenticity of herbal products. Another marker, protein A, was identified and shown to be comparable to eurycomanone in its ability to differentiate between TA- and non-TA-containing products. Both markers are quantitative and they utilize different characterization features and tools. The protein in Marker A has the advantage that it can be developed as a simple biosensor (similar to point-of-care testing), or an advanced biosensor (mass-spectrometry-related; SELDI TOF MS, MALDI TOF MS, LC-MS, etc). Hence, it is expected that the development of TA-based products worldwide can be improved using eurycomanone and protein marker as complementary markers for improved product standardization, quality control and authentication.


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