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Review

A Multifaceted Review of *Eurycoma longifolia* Nutraceutical Bioactives: Production, Extraction, and Analysis in Drugs and Biofluids

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ABSTRACT: *Eurycoma longifolia* Jack (known as Tongkat Ali) is a popular traditional herbal medicine, native to southeast Asia, that is well-known for its aphrodisiac as well as several other effects. Mostly, the root extract of *E. longifolia* is used as a folk medicine for sexual dysfunction, aging, anxiety, exercise recovery, fever, increased energy, and osteoporosis. These health effects led to the inclusion of *E. longifolia* in dietary supplements, particularly for bodybuilding purposes. These effects are mediated by a myriad of bioactive compounds belonging to quassinoids, canthin-6-one alkaloids, tirucallane triterpenes, squalene derivatives, and bioactive steroids. Among these phytoconstituents, quassinoids account for a large portion of *E. longifolia* root phytochemicals. Of these ingredients, eurycomanone, the major quassinoid in *E. longifolia* extract, accounts to a large extent for its health effects. This review capitalizes on the novel trends toward the production of *E. longifolia* bioactives using biotechnology and extraction optimization for best yields and recovery. Alongside, novel extraction methods, i.e., green techniques, of *E. longifolia* bioactives are described. Further, an overview of the different analytical approaches for the quality control assessment of *E. longifolia* plant material and nutraceuticals is presented alongside studies in body fluids to determine its pharmacokinetics and efficacy level. Such a compilation of analytical methods will help ensure safety and efficacy of that major drug.

1. INTRODUCTION

Eurycoma longifolia Jack is a well-known tropical herb belonging to the Simaroubaceae family. *E. longifolia* is indigenous to southeast Asia including Indonesia, Malaysia, and Vietnam. Some species are also found in regions of Myanmar, Cambodia, and Thailand. This plant is commonly known in Malaysia as Tongkat Ali, meaning "walking stick" and denoting the presence of long twisted roots. Other synonyms include Ali's Umbrella or Malaysia Ginseng (Malaysia), Iandon (Thailand), Cay ba benh (Vietnam), Pasak Bumi or Bedara Pahit (Indonesia), and tho nan (Laotian).[1]

The root extract of Tongkat Ali has long been used in traditional medicine for enhancing testosterone levels in men. Hence, increased interest in the regular intake of root extracts has developed among individuals to enhance muscle mass and strength for body building. Moreover, additional uses (particularly the roots) in indigenous traditional medicines include antipyretic, cytotoxic, antimalarial, antiulcer, and aphrodisiac properties.[2] Root extracts have been used traditionally to reduce blood pressure and fatigue, as well as treatment of dropsy, cough, diarrhea, dysentery, glandular swelling, and bleeding.[2] *E. longifolia* decoction is used as a tonic for women after childbirth and to increase vitality and energy for men.[2] In contemporary dietary supplements, Tongkat Ali is included to restore hormonal balance (cortisol/testosterone levels), improve libido and energy, and enhance weight loss and sports performance.[3] A wide array of phytoconstituents has been isolated and characterized from *E. longifolia*, particularly from the roots, including β-carboline alkaloids, canthin-6-one alkaloids, quassinoids, quassinoid diterpenoids, squalene derivatives, tirucallane-type triterpenes, biphenylneolignans, laurycolactone, eurycolactone, and eurycomalactone.[1,4] Major isolated chemical constituents from *E. longifolia* Jack are illustrated in Figure 1. The safety and efficacy of Tongkat Ali depend to a great extent on the concentration of their bioactive constituents warranting the development of analytical tools for its accurate quality control and or adulteration detection for such a valuable drug.

Promising biotechnological approaches such as cell and organ cultures allow for the production of scarce and less cultivated plant bioactives[5,6] as in the case of Tongkat Ali. The production of *E. longifolia* bioactives, *i.e.*, alkaloids, phenolics, and flavonoids from hairy roots or calli have been developed,[7] among which hairy root culture seems beneficial for the

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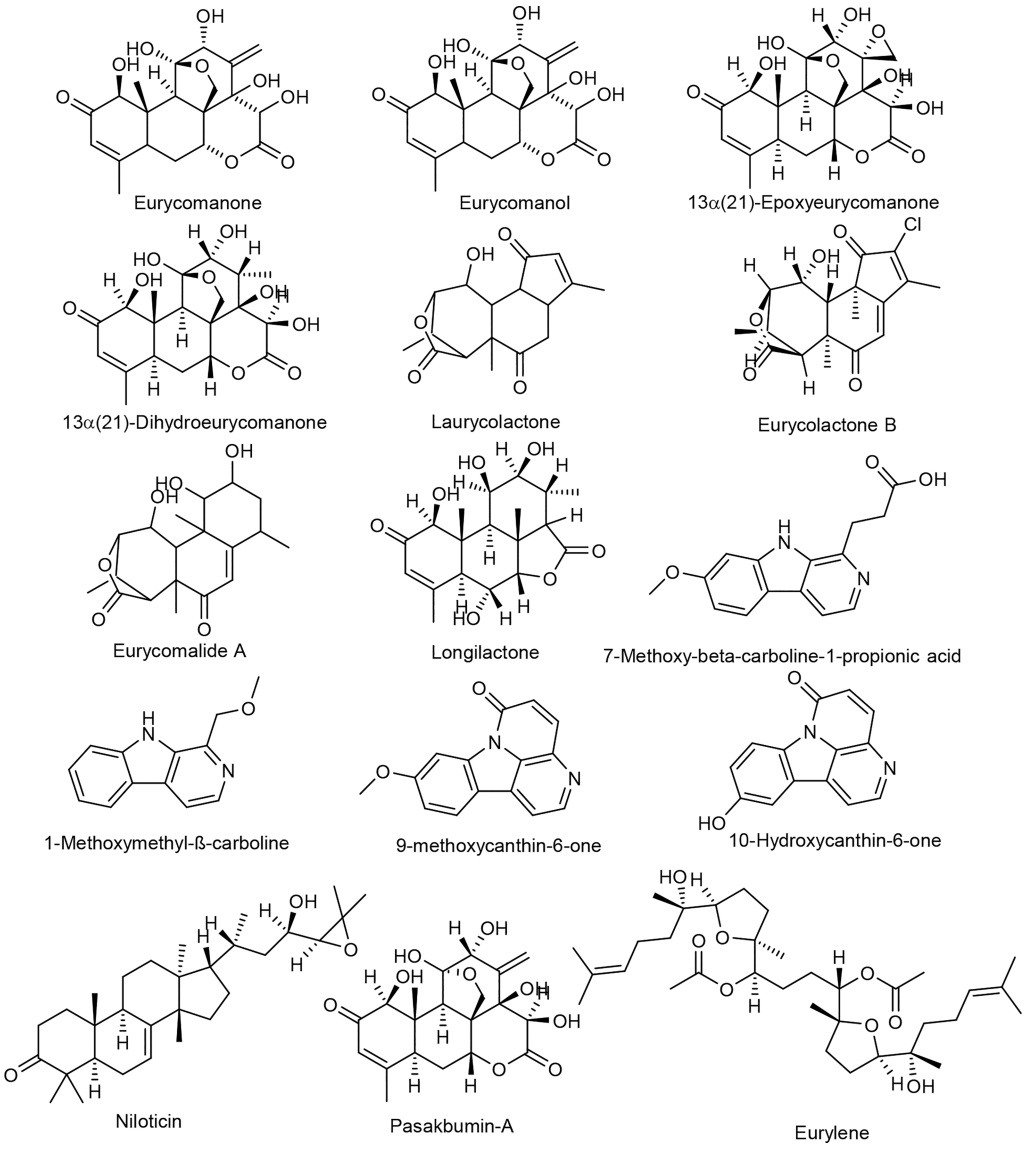

Figure 1. Representative major phytochemicals from *E. longifolia* Jack.

production of bioactives at much higher levels than the original plant.[8]

This review details the different biotechnological techniques that have been exploited in the literature and reported for the optimal production of bioactive compounds from *E. longifolia* targeting its different classes such as phenolics and flavonoids, and quassinoids, i.e., eurycomanone.

2. *EURYCOMA LONGIFOLIA* AS A NUTRACEUTICAL IN MARKETS WORLDWIDE

All parts of the plant have been used for various health purposes with the root being the most used. Products that encompass *E. longifolia* as the main ingredient have been reported with several indications. There are several commercial products in the market containing Tongkat Ali. A commercial *E. longifolia* infused coffee is marketed as a powdered dark brown beverage which contains *E. longifolia* roots. It is a premixed instant coffee powder with a unique taste and aromatic sensation indicated to boost wellness.[9] Physta, the world's first patented Tongkat Ali extract, also known as LJ100 in the United States is a dried extract powder from roots produced by freeze-drying to preserve nutrients and avoid deterioration during extraction,[10,11] typically standardized to contain 1.5% eurycomanone. This product has been indicated to deliver the highest level of bioactive substances from the root, with maximum potency, safety, and health benefits.[10]

Being mostly grown in Malaysia, several products of *E. longifolia* are sold in that market mostly as instant coffee. A mixed product of *E. longifolia* and ginseng extracts alongside other ingredients is indicated to act as an energizer and immunity enhancer.[3] A popular coffee product containing instant Tongkat Ali and ginseng along with other ingredients is indicated to strengthen immunity.[1] A flavored energy drink combining instant Tongkat Ali and ginseng extracts, alongside vitamin B6 and B12, is regarded as an energy drink.[12]

A dietary supplement in the form of capsules is also produced from *E. longifolia* extract likewise standardized for its eurycomanones that account for most of its effects. It has been indicated to improve libido and support healthy cortisol levels leading to mood improvement.[13] A similar pharmaceutical preparation produced to encompass a 300 mg extract in the form of tablets is indicated for enhancing testosterone levels

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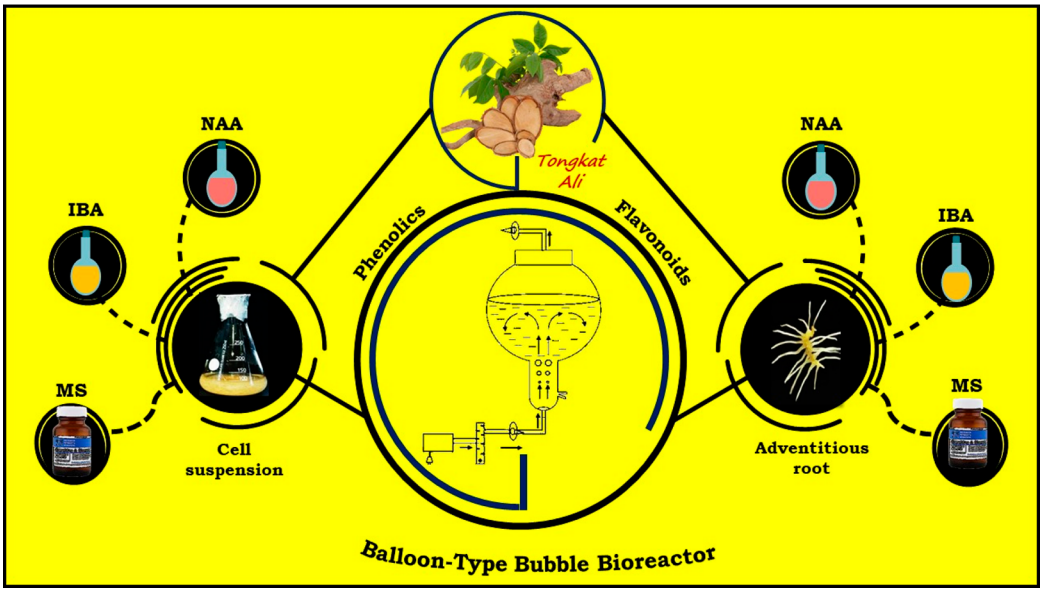

Figure 2. Schematic representation summarizes the biotechnological techniques employed in the production of phenolics and flavonoids from adventitious roots and cell suspension culture of *E. longifolia* in a balloon type bubble bioreactor using indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA) as growth regulators and Murashige and Skoog (MS) medium.

while improving male fertility, relieving stress, increasing energy levels and muscle strength, and boosting athletic performance at a recommended daily dosage of one to two tablets daily.[14]

Another nutraceutical is made up of *E. longifolia* extract powder to encompass a root extract at a higher dose level of 700 mg, with several indications, i.e., to support growth of healthy tissues, immunity, strength and endurance levels, mental wellness and brain function, as well as healthy and strong bones.[15]

3. NOVEL PRODUCTION TOOLS OF *E. LONGIFOLIA* BIOACTIVES USING BIOTECHNOLOGY

E. longifolia is an endangered plant and has been declared in most of the countries as a protected plant. Alongside, its harvesting has become highly restricted in nature.[8] Besides, the production of *E. longifolia* roots is often challenging and varies with seasons and or environmental conditions. Additionally, harvesting of *E. longifolia* roots takes place after 4−7 years of cultivation warranting for the development of other approaches for the production of its root bioactives to meet the increasing demand for its inclusion in nutraceuticals.[8] Promising biotechnological approaches such as cell and organ cultures allow for the production of scarce and strenuously cultivated plant bioactives,[5,6] and has also been reported in that plant. The production of *E. longifolia* several bioactives, i.e., alkaloids, phenolics, and flavonoids from hairy roots or calli, have been developed.[7]

Being a root, hairy root culture has been used to produce secondary metabolites from *E. longifolia* as a good model to improve metabolites production.[16] Hairy root culture is typically generated by transforming plant cells using *Agrobacterium rhizogenes* to produce secondary metabolites with their genetic and biochemical stability. Reports revealed that hairy roots are beneficial for producing bioactive compounds at much higher levels than that originally in the plant due to their rapid rate of growth. Adventitious root cultures can also be used to produce secondary metabolites at a large scale being more efficient than conventional process of culturing.[8] Application of exogenous abiotic and biotic elicitors can further stimulate defense responses related to stress in plant cells to improve the yield of secondary metabolites in tissue cultures.[17,18] This review details the different biotechnological techniques that have been exploited and reported for the optimal production of bioactive compounds from *E. longifolia* targeting its different classes such as phenolics and flavonoids, quassinoids, i.e., eurycomanone, as illustrated in the next subsections.

3.1. Production of Phenolics and Flavonoids from *E. longifolia* Using Elicitors in Adventitious Roots. Adventitious root culture employs the use of a large-scale bioreactor culture to produce secondary metabolites commercially. This process is more efficient than traditional cultures, being more stable and ensuring steady production of secondary metabolites.[8] A liquid medium having forced aeration to help the rate of growth is used for the bioreactor culture and to further optimize for conditions in a bioreactor culture, i.e., temperature, pH, oxygen, carbon dioxide, and nutrient level, to ensure best levels of metabolites.[19] Lulu et al.[8] investigated the effect of auxin of different types and levels and the ratio of NH_4^+:NO_3^- on the production of root biomass and secondary metabolites level from *E. longifolia* adventitious roots. An overview is provided in Figure 2.

Different levels of indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA) to grow adventitious roots of *E. longifolia*[20] were tested grown in a 3 L balloon-type bubble bioreactor (BTBB). Maximum fresh and dry weights of 77.6 g/L and 7.2 g/L was observed with 7.0 mg/L IBA with the increase being proportional to increasing NAA levels up to 5 mg/L. Phenolic and flavonoid contents also increased with IBA concentration, with the highest levels observed at 7.0 mg/L IBA and 5.0 mg/L NAA. Likewise, the effect of inorganic nitrogen sources, i.e., KNO_3 and NH_4NO_3, on the growth of adventitious *E. longifolia* root was studied, at a ratio of 15:30 (NH_4^+:NO_3^-) showing the highest biomass yield. The ratio of NH_4^+:NO_3^- influenced secondary metabolites production,

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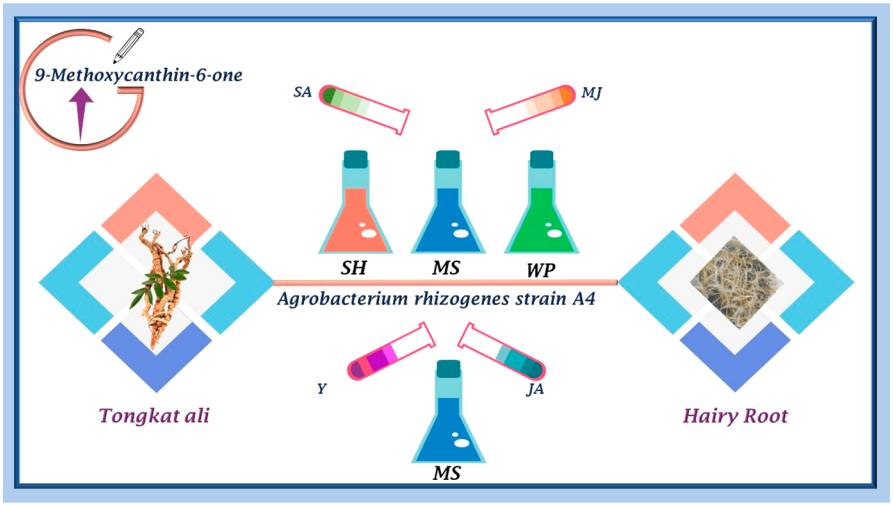

Figure 3. Schematic representation summarizing the biotechnological techniques in production of 9-methoxycanthin-6-one in hairy root culture of *E. longifolia* after root transformation with *Agrobacterium rhizogenes* strain A4. Jasmonic acid (JA), methyl jasmonate (MJ), salicyclic acid (SA), and yeast (Y) served as elicitors, and different basal media were used including Murashige and Skoog (MS), Shenck and Hildebrandt (SH), and McCown's woody plant (WP).

with a maximum level achieved at a ratio of 1:2; the production of phenolics and flavonoids was found to be at 38.59 and 11.27 mg/L medium, respectively. Hence, a ratio 1:2 of NH_4^+:NO_3^- is suggested to be suitable for secondary metabolites production in *E. longifolia* adventitious roots in a bioreactor culture system and has yet to be examined in other growth systems. The comparison between growth rate using a flask culture and bioreactor culture was carried out under same conditions, with noted improved production of bioactive compounds after 7 weeks observed in a bioreactor culture as opposed to the shake flask culture with maximum levels of phenolic and flavonoids being at 128.0 and 33.8 mg/L medium, respectively.[8] Interestingly, no report has been made on changes in quassinoid levels exemplified by eurycomanone as a major bioactive in *E. longifolia* under these conditions and should be examined in the future.

3.2. Production of Phenolics and Flavonoids from Cell Suspension Cultures in a Balloon Type Bubble Bioreactor Using Elicitors. Shim et al.[6] produced bioactive compounds from *E. longifolia* cell suspension cultures in BTBB with optimization of inoculum density and aeration rate bioprocess parameters. Optimization of medium parameters such as the effect of auxins (NAA and IBA), MS salt strength, sucrose, and nitrogen levels to produce biomass was initially tested, revealing that supplementing MS medium with NAA was better than IBA to yield the highest biomass. Figure 2 provides an overview of *E. longifolia* culture optimization in the context of production of phenolics and flavonoids from adventitious roots and cell suspension culture.

The low and high salt strengths were found to be not suited for biomass production, whereas full-strength MS medium provided optimum yield. A sucrose concentration at 3% was suitable for production of biomass, with the highest biomass achieved at a ratio of 0:60 NH_4^+: NO_3^-. Further effects of inoculum density (40, 50, 60, 70, and 80 g/L) and aeration rate of 0.05, 0.1, 0.2, and 0.3 were optimized for bioactive compounds yield. Maximum levels of phenolics and flavonoids of 7.7 and 1.7 mg/g were observed at an inoculum density of 50 g/L. Nevertheless, no report so far has attempted to monitoring change in quassinoids exemplified by eurycomanone as a major bioactive in *E. longifolia* under these conditions, and this should further be examined in the future.

Increased air supply at an aeration rate of 0.5 vvm (air volume per culture volume) weekly led to a decrease in root mass though with increased accumulation of phenolics and flavonoids at 10.3 mg/g and 3.8 mg/with increasing air supply. Highest recovery of phenolics and flavonoids from *E. longifolia* cell biomass was achieved using 60% ethanol compared to water and methanol at a yield of 11.8 mg/g and 3.2 mg/g, respectively[6] and suggestive that such solvent proportion provides the best recovery rate.

3.3. Effect of Elicitors on Bioactives in Hairy Root Culture. In a study using hairy root culture of *E. longifolia*,[16] 9-methoxycanthin-6-one, an indole alkaloid that is the 9-methoxy derivative of canthin-6-one, was detected. *E. longifolia* root was transformed with *Agrobacterium rhizogenes* strain A4 to initiate hairy roots growth, further elicited with methyl jasmonate (MeJA) and salicyclic acid (SA) compared to control as depicted in Figure 3. Hairy roots showed maximal growth at the 10th week compared to cell suspension cultures showing a maximum growth at 3 weeks. Elicitation with MeJA and SA both resulted in an early decrease in biomass at high levels at 24 h, and with no further growth reduction at 10 weeks. Despite reduction in biomass, 9-methoxycanthin-6-one showed a 3-fold increase in elicited hairy root compared with control. The factors responsible for a decline in growth of elicited hairy roots were not determined as part of this study.[16] Maximal increase in 9-methoxycanthin-6-one was observed at a dose of 0.1 mM for both MeJA and SA, with a decline at higher doses.[16]

The elicited culture media (4- and 10-weeks old) of both elicitors encompassed higher levels of 9-methoxycanthin-6-one than control, albeit the 10th-week elicited medium showed a 1.5 to 2 times fold increase than the fourth-week medium. Hence, elicitor dose, timing, and duration of elicitation appeared as variables that need to be optimized for optimum production of 9-methoxycanthin-6-one.[16] How other bioactives respond to elicitation, i.e., flavonoids and phenolics, was not examined as part of this study and should now follow.

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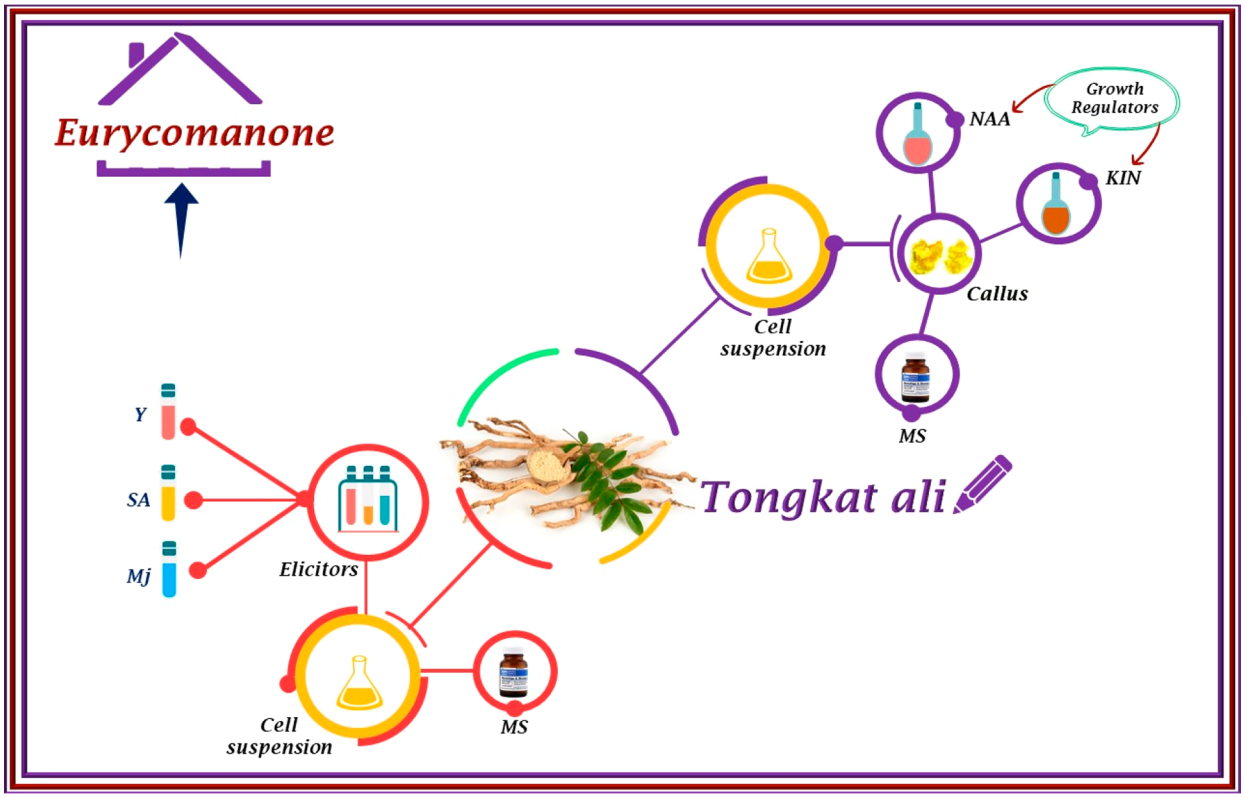

Figure 4. Schematic representation showing biotechnological techniques in the production of eurycomanone in cell suspension culture of Tongkat Ali using methyl jasmonate (MeJa), salicylic acid (SA), and yeast (Y) as elicitors, Murashige and Skoog (MS) as a medium, and Kinetin (KIN) and naphthalene acetic acid (NAA) as growth regulators.

3.4. Optimization of Medium and Production of Bioactives Using Different Basal Media and Elicitation of Hairy Root with Jasmonic Acid and Yeast Extract. Tran et al.[18] investigated optimum medium composition and elicitation for the production of 9-methoxycanthin-6-one using different basal media including Murashige and Skoog (MS), Shenck and Hildebrandt (SH) and McCown's woody plant (WP) to grow the hairy root culture of *E. longifolia*. Effect of elicitors on the secondary metabolites accumulation was examined including jasmonic acid (JA) and yeast extract (YE).

Maximum biomass was observed in case of SH medium (0.79g DW) followed by MS (0.67g DW) and WP (0.59 g DW). With regards to 9-methoxycanthin-6-one production level, a lag phase was observed with highest value of 0.429% on a dry weight basis in an exponential phase after 25 days, and to further decline post 30 days to reach 0.24%. In case of SH and MS media, a lengthy lag phase was observed with highest levels of 0.133 and 0.145% after 20 and 25 days, respectively confirming that WP medium provided the best yield of 9-methoxycanthin-6-one accumulation in hairy root culture, though with highest biomass observed in SH medium. Whether such medium effect is also observed in other culture types i.e., cell culture ought to be examined to be conclusive. The highest level of 9-methoxycanthin-6-one in hairy root culture grown in WP medium may be attributed to the high sulfate content of the medium at 7.34 mM compared to MS and SH media containing *ca.* 1.75 mM and 1.69 mM respectively.[18]

With regard to a further elicitation effect, it was reported that JA at a high dose level of 16 mg/L resulted in a decrease in 9-methoxycanthin-6-one *ca.* a two fold decrease, while the lower dose at 8 mg/L resulted in a 2.6-fold increase, and suggestive of the need to optimize for elicitors dose. Whether the lower induction level of JA compared to MeJA in the study of Nhan and Loc[21] is attributed to MeJA improved cell permeability being less polar then JA is likely to account for such differences, or it is attributed to the different experimental setup. Treatment with 20 and 40 mg/L YE resulted in *ca.* a 2- and 4-fold increase in 9-methoxycanthin-6-one accumulation after 25 days compared to control, while at a higher dose of 80 mg/L accumulation of 9-methoxycanthin-6-one showed inhibition. Consequently, 40 mg/L YE was reported to be a better elicitor than 8 mg/L jasmonic acid, and whether a synergized effect be observed upon combining more than one elicitor, i.e., JA and SA have yet to be reported. How SA and JA signals the increased production of 9-methoxycanthin-6-one inside cells should be pursued in the future. These results confirm that hairy roots can provide a better source for the recovery of quassinoids than normal roots especially with elicitation, i.e., YE.

3.5. Optimization of Growth Parameters for Production of Bioactives in Callus and Cell Suspension Culture, and Post Elicitation. Optimization of growth media composition was assessed in Nhan and Loc[7] in the context of cell biomass and eurycomanone production. Callus culture of *E. longifolia* callus was moved to an MS medium containing carbon sources (fructose, sucrose, and glucose) as well as various concentrations of plant growth regulators (NAA and Kinetin-KIN) and subcultured every 2 weeks in new medium. An overview is provided in Figure 4. Results revealed that after 2 weeks of culturing using plant growth regulators, NAA yielded the highest efficiency at 1.25 mg/L with callus

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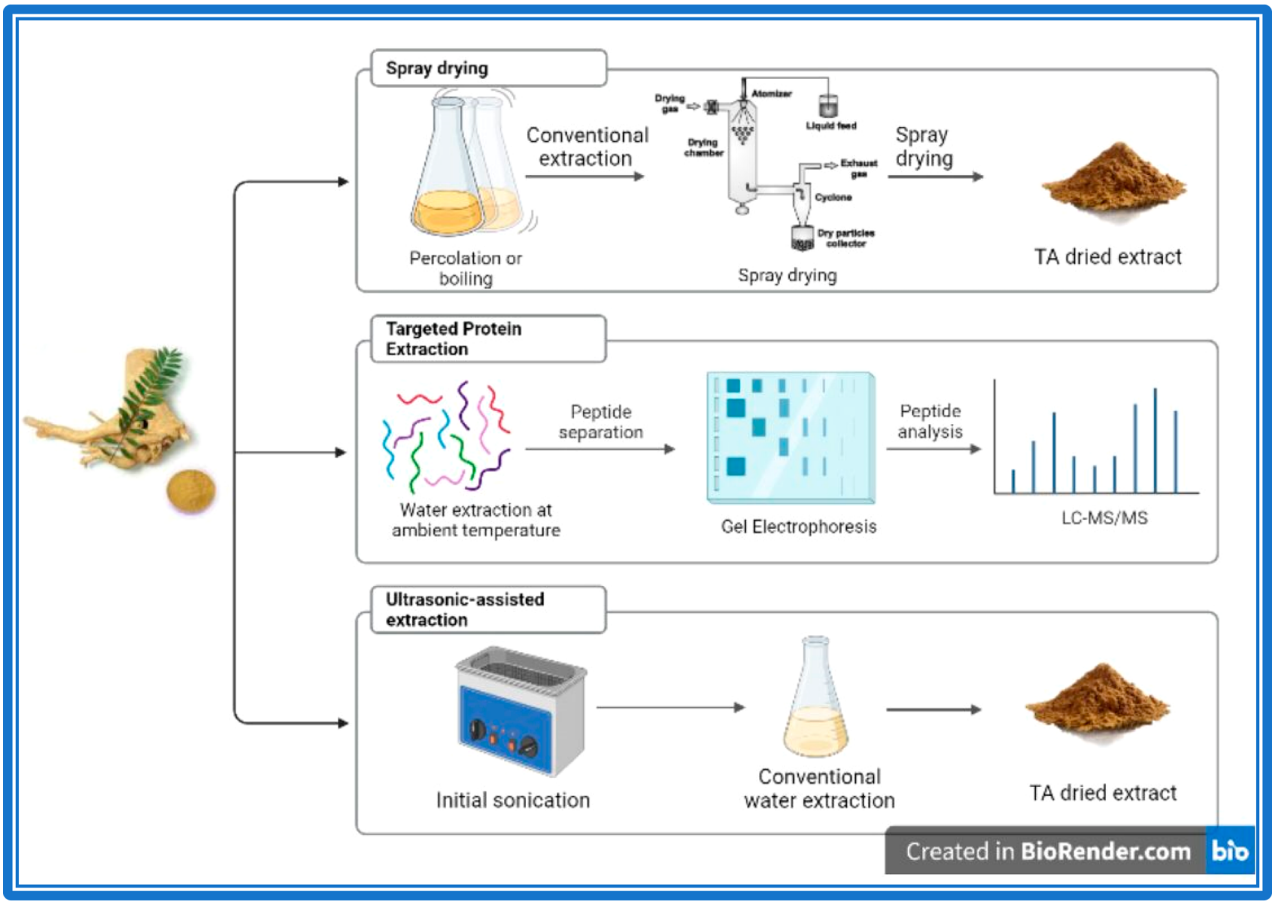

Figure 5. Extraction techniques of *E. longifolia* bioactives.

biomass from 3 g of inoculum to reach 23.59 g, whereas the lowest callus growth was observed at a NAA dose between 2 and 2.25 mg/L. The maximum callus biomass was recorded at 30−32 g using 1.25 mg/L NAA and 0.75−1 mg/L KIN suggesting that the combination of NAA and KIN promoted callus growth compared to only NAA.

With regard to bioactives yield in callus, eurycomanone showed a maximal level of 1.7 mg/g dry weight on the 14th day at a comparable level to that of 2.1 mg/g detected in a root of a 5-year-old tree and suggestive that it can serve as a source of this drug aside from the ease of extraction from cell culture compared to plant material.[7] Likewise, Siregar et al.[22] reported a 5 times increase in callus mass of *E. longifolia* grown on MS medium supplemented with 1 mg/L NAA, while a maximum biomass yield was observed using modified MS medium supplemented with 2 mg/L NaH_2PO_4.[22]

Carbon sources have been reported to influence the callus growth and further phytochemicals production, with sucrose having the greatest effect on cell growth and accumulation of eurycomanone compared to glucose, while no increase in growth was observed when fructose was used as a source of carbon in the media. Sucrose level of 20−40 g/L showed a significant change ($p < 0.05$) on dry cell biomass compared to control (17−18 g, $p > 0.05$), with 30 g/L having maximum biomass concurrent with the highest eurycomanone level of 1.7 mg/g.[7]

Compared to optimization attempted in callus culture described in the previous section, similar attempts were reported in liquid cell suspension with improved production of eurycomanone using elicitation in cell suspension culture. Biotic and abiotic elicitors are extensively reported in the literature for the activation of natural products biosynthesis including the phytohormones MeJa and SA.[23] Cell suspension culture was developed previously[7] by agitating a callus in a medium containing basal MS medium with 30 g/L sucrose, 1.25 mg/L NAA, and 1 mg/L KIN. Elicitation was achieved using yeast extract (YE) (20−250 mg/L), MeJA (10−500 μM), and SA (10−500 μM) in the medium. Elicitation at the beginning of the culture led to cell growth inhibition with a 0.4−0.5-fold decrease in the case of YE and SA elicitation, whereas MeJA showed the strongest inhibition with a 0.9-fold decrease in cell growth compared to control at the highest dose level of 500 μM. Nevertheless, it should be noted that such a decrease in growth was paralleled by an increase in eurycomanone detected at a 2 fold higher level than control in the case of YE 200 mg/L, 2 fold with 20 μM SA, and 4 fold with 20 μM MeJA. These results suggest that among elicitors MeJA is the best elicitor to upregulate quassinoids biosynthesis in *E. longifolia* cell culture. It has yet to be observed the response of other metabolite classes to be conclusive and the molecular mechanisms underlying such changes. Optimization of cell growth while increasing production of targeted bioactive should thus follow based on these results if it is to be applied at large scale for commercial production.[21]

The elicitation time at these optimal doses of elicitors was used to identify the appropriate harvest times at which maximum eurycomanone production was observed. In the case of 20 μM MeJA, a maximum eurycomanone level of 17.36 mg/

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g was achieved on fourth day post elicitation with 20 μM MeJA at *ca.* 10 times higher than that of untreated cells and 5-year-old tree roots.[7] In contrast, in case of 20 μM SA and 200 mg/L yeast extract, maximum eurycomanone level of 5.2 and 6.25 mg/g (1.46 and 1.69 times higher than control), respectively was observed at fourth or sixth days. These results conclude that best harvest time for eurycomanone from cells post elicitation is from 4 to 6 days, with MeJA being the most effective.[21]

4. OPTIMIZATION AND NOVEL EXTRACTION METHODS, I.E., GREEN TECHNIQUES OF *E. LONGIFOLIA* BIOACTIVES

Nowadays, herbal-based phytochemical industry is considered an important industrial sector. However, a common drawback often encountered is the application of traditional methods (e.g., percolation or boiling) in the large-scale production of these phytochemicals. The main obstacle encountered is the low overall yield of *E. longifolia* extracts and major losses of bioactives.[24] Hence, development of this herbal-based industry into a more profitable industrial sector is crucial.[24]

E. longifolia water extract represents a valuable product in the phytochemical industry, processing of which needs to be efficient to meet its increasing demand. Several attempts were carried out to optimize the extraction of *E. longifolia* bioactives (Figure 5). Conventional extraction methods of *E. longifolia* include high pressure water extraction, where the roots are boiled as a decoction, then subjected to subsequent spray drying for the finishing step to produce good quality dry particles.[25] Harun et al.[26] investigated the optimum process parameters for the spray drying of Tongkat Ali extracts. Results revealed that the optimum conditions were at a feeding temperature of 25 °C, air inlet temperature of 160 °C, feed flow rate of 4.86 mL/min, and air pressure of 17.91 psi.[26]

Athimulam et al. developed several optimizations and debottle necking strategies to optimize the production of *E. longifolia* water extract using the commercial batch process simulator SuperPro Designer, a window-based simulation software. This software is utilized for modeling food, pharmaceuticals, biochemicals, specialty chemicals, as well as other manufacturing processes.[24] Four alternative production schemes were proposed. A pilot scale production scheme was used to simulate the base case process with a 390 kg annual production rate of *E. longifolia* extract. In the first proposed scheme, a new spray drying procedure was added in parallel with the existing spray dryer to reduce the spray drying operation process time by half. Scheme two allowed water extract concentration prior to spray drying via a new double effect forward feed evaporator reducing the process time for the spray drying operation by half. Scheme three combined the strategies in the first two schemes reducing the process time of the spray drying operation from 22.11 to 5.17 h. All three debottlenecking schemes demonstrated significant improvement on the annual production, with scheme three exhibiting the highest annual production rate. However, these three schemes were still economically infeasible owing to low ROI values below the desired 30% despite increased annual throughput. Thus, a new grinder and packaging section were proposed. The final alternative scheme was reported to achieve a 3.00% product yield with 1137 kg of *E. longifolia* extract annual production. Athimulam et al. succeeded in reducing the minimum batch cycle time from 24.4 to 8.3 h. Besides, economic analysis revealed an annual revenue of $6.32 M for the proposed alternative production scheme with a gross margin of 86% and a 55% return on investment.[24]

Moreover, several extraction procedures were reported targeting various peptides in *E. longifolia* roots harvested from Perak and Pahang in Malaysia.[27] depending on the type of targeted protein. Utilizing water as an extraction solvent at ambient temperature helps get rid of plant pigments, lipids, and phenolics which might interfere with consequent protein analysis and contaminate the protein extract. This protocol produced superior protein yield as determined using gel electrophoresis (SDS-PAGE). Two protein spots for Tongkat Ali Perak (49.8 and 5.5 kDa) and four protein spots for Tongkat Ali Pahang (49.8, 24.7, 21.1, and 5.5 kDa) were obtained.[27]

Bolong et al. attempted to isolate and concentrate the targeted 4.3 kDa peptide fraction, the aphrodisiac marker, from *E. longifolia* water extracts using hollow fiber membranes made of poly(ether sulfone) (PES) and modified by negatively charged-modifying macromolecules. This resulted in a permeate which was 10 times concentrated compared to the actual overall extract.[28] In the same context, another study was conducted by Suan Chua et al. with four extraction methods carried out to extract the plant protein from *E. longifolia* roots harvested from Perak and Pahang, Malaysia. Results revealed that water extraction produced a higher protein content (13−29 mg/g pellet), but at a lower yield (0.1% w/w) compared to precipitation methods. Meanwhile, higher molecular weight hydrophobic proteins (46−51 kDa) were detected only in the TCA acetone and phenol-SDS. The optimization of the processing parameters (air inlet temperature, feed temperature, and feed flow rate and air pressure) for spray drying of *E. longifolia* extract was performed using RSM of the Box-Benhken design and simulated using Design Expert software. The optimum condition achieved for *E. longifolia* spray drying was at an air inlet temperature of 160 °C, feed temperature of 25 °C, air pressure of 17.91 psi, and feed flow rate of 4.8 mL/min, which has yet to be implemented at a larger scale.[29] Thus, it is quite clear that different extraction methods recover different kinds of plant proteins.

Ultrasonic-assisted extraction is a green extraction technology that could be applied to increase the yield of targeted phytochemicals[30] and allows minimization of product wastes and maintenance costs[31] besides reducing environmental impacts.[32] Abugabr Elhag et al.[31] investigated the optimization of protein extraction from *E. longifolia* roots by ultrasonic-assisted extraction together with mechanical agitation. Results revealed that applying eccentric agitation accompanied by ultrasonic-assisted extraction allowed protein extraction in a short time. A Central composite design (CCD) was employed to optimize ultrasonic-assisted water extraction of proteins from *E. longifolia* roots. Response surface methodology (RSM) was applied to evaluate the effects of five independent variables including particle sizes, extraction temperatures, agitation speeds, amplitude, and duty cycle.[31] Nevertheless, further studies are required to provide deeper insights to develop an optimum setup that could be applicable at an industrial scale.

A sequential extraction method was employed to extract saponins from *E. longifolia* roots. Elhag et al.[33] investigated the optimum conditions to extract saponins by both conventional water extraction and nonconventional ultrasound-assisted extraction to maximize saponin yields. The selected optimum conditions were further employed to establish a sequential extraction process to increase yield with less extraction time

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and avoiding saponins degradation. Central composite designs (CCDs) for ultrasound-assisted extraction and water extraction were examined, with results revealing that the agitation speed acts as an influencing factor where higher agitation speeds increased saponins yield in water extraction; meanwhile, lower agitation speed ranges were preferred in ultrasound-assisted extraction. The study suggested an initial sonication for 5 to 10 min followed by conventional water extraction for 20−25 min.[33]

A dimensionless model was proposed for scaling-up the solid−liquid extraction of *E. longifolia* roots. Harun et al.[34] concluded that the best fit of all models was $ShSc^{-1}$ dimensionless number. At optimum conditions for the extraction process, a solvent to raw material ratio of 12.5:1, duration of extraction of 53 min, roots particle size of 0.5 to 1.0 mm, and the extract yield of 8.76% at lab-scale, the $ShSc^{-1}$ no. was found to be 0.0312. This study provided useful scale-up knowledge to ensure a smooth transition from the lab-scale to pilot-scale.[34]

5. ANALYSIS AND QUALITY CONTROL APPROACHES OF *E. LONGIFOLIA* NUTRACEUTICALS

Quality control of herbal based products is a crucial step toward implementation of phytomedicine and its integration in primary healthcare. Alongside, the safety and efficacy of herbal medicines depend to a great extent on the concentration of their bioactive constituents. Thus, fast and effective analyses approaches are essential to monitor the inherent characteristics of herbal medicines and their corresponding extracts and pharmaceutical products.

5.1. Chemical Analysis on *E. longifolia* Nutraceuticals. Sometimes, herbal based products may reveal the absence of the herbal ingredient as claimed in the product label. Thus, it is crucial to develop a fast, reliable, and sensitive method to analyze herbs and their products. Like other plant-derived nutraceuticals, *E. longifolia* roots were used as the sample matrix. The marker compound, eurycomanone, the major quassinoid, was extracted using a sonication assisted extraction method. Quantitative analysis was then performed using high throughput ultra-performance liquid chromatography-tandem mass spectrometry (UPLC−MS/MS) employing multiple reaction monitoring (MRM) as a selective technique. The positive ion transitions for eurycomanone were m/z 409 → 391 in MRM. This method was further validated for its accuracy, precision, linearity, robustness, detection, and quantitation limits.[35]

Furthermore, another method simultaneously quantified six major quassinoids in *E. longifolia* roots using liquid chromatography coupled to tandem mass spectrometry. Eurycomanone, 13,21-dihydroeurycomanone, eurycomalactone, longilactone, 13α(21)-epoxyeurycomanone, and 14,15β-dihydroxyklaineanone contents were assessed in Tongkat Ali containing dietary supplement capsules or tablets. Eurycomanone was found to be the most abundant quassinoid in all tested samples. This method could be applied for the authenticity verification and quality control of Tongkat Ali containing dietary supplements.[36]

An online solid phase-extraction liquid chromatography (SPE-LC) approach was applied for acquiring a chromatographic fingerprint of Tongkat Ali roots and its products combined with chemometric tools. The quality of 17 *E. longifolia* roots and 10 commercial products (capsules) was evaluated. The acquired chromatographic data set (using 37 selected peaks) was further subjected to chemometric modeling, including cluster analysis (CA), and principal component analysis-discriminant analysis (PCA-DA). The samples were grouped based on their quality into three clusters. No significant difference was spotted between the roots and products within the same cluster as displayed by pattern matching analysis. The combination of chromatographic fingerprint and chemometric tools could provide a comprehensive evaluation for the efficient quality control of Tongkat Ali herbal formulations.[37]

^{1}HNMR-based discriminatory analysis also can be applied for the quantification of quassinoids, the major secondary metabolites of *E. longifolia* roots.[38] A phytochemical profile was established to identify the primary metabolites as well as major quassinoids: eurycomanone, eurycomanol, 13,21-dihydroeurycomanone, and eurycomanol-2-*O*-β-D-glycopyranoside. In addition, quassinoids were quantified using external calibration curves. Besides, *E. longifolia* roots from different locations were efficiently discriminated based on their metabolic profile acquired using ^{1}H NMR spectra targeting quassinoids;[38] whether such pattern is present for other classes should be examined.

5.2. Safety Evaluations on *E. longifolia* Nutraceuticals. Recently, fraudulent product counterfeiting has been targeting these herbal aphrodisiac preparations. Besides, eurycomanone is suspected to exhibit toxic effects at higher levels. In this context, a highly selective HPLC-DAD/ELSD method has been developed to assess the quality of commercially available Tongkat Ali containing products. A mixture of 27 reference compounds was used for qualitative issues and was validated for the quantification of three quassinoids, namely, eurycomanone, laurycolactone A, and longilactone. Calibration curves were constructed. Eight products were randomly purchased and analyzed. Only five products contained detectable amounts of *E. longifolia* compounds. Eurycomanone levels varied from 0.22 ± 0.002 mg to 1.84 ± 0.08 mg eurycomanone per capsule, corresponding to 0.76 ± 0.02−31.90 ± 0.21 mg maximal recommended daily intake.[39]

The identity of natural products could be effectively confirmed by quantifying the phytoconstituents using infrared absorption spectrophotometry and allocating the characteristic absorption bands in the acquired spectrum.[40,41] A dual drug screening method comprising a "dedicated" near-infrared spectral (NIR) database of common medicines in addition to a "unified" database was established to identify the sildenafil analogue in *E. longifolia* products. Diffuse reflectance spectra were acquired for 10 commercial herbal products containing *E. longifolia* over a 1100−2500 nm wavelength range. Two products displayed a similarity index of more than 0.1 in a spectral search against an *E. longifolia* products' dedicated database indicating significantly different spectra. Accordingly, further spectral searches against the unified database displayed a close match to the spectra of sildenafil citrate suggesting the presence of a sildenafil analogue which was further confirmed by clustering of these spectra in the PCA score plot. It could be concluded that this approach can be used to detect adulteration of foods and drugs in the absence of a reference product or standard active ingredient.[42]

Infrared spectroscopy has been applied to acquire unique fingerprints of *E. longifolia* and its successive extracts (hexane, ethyl acetate, dichloromethane, and methanol).[43] Adib et al. used FT-IR, second derivative infrared spectroscopy, and two-

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dimensional correlation infrared spectroscopy (2D-IR) for the analysis of different *E. longifolia* extracts. Results indicated that FT-IR and 2D-IR could provide structural information on the main components in the samples' complicated system revealing a large amount of quassinoids exhibiting characteristic absorption peaks at 1700 cm^{-1}, 1670 cm^{-1}, 1600 cm^{-1}, 1500 cm^{-1}, and 1270 cm^{-1}. Moreover, this method could distinguish minor differences between systems showing similarity in the macro-fingerprint characters. Thus, this method could be implemented for nutraceuticals QC offering rapid, accurate, effective, and reproducible results.[43]

Azlah et al. applied FTIR spectroscopy to scan the dried and ground leaves of several herbal plants. The stretching and vibration behavior of functional groups was investigated at different temperatures ranging from 20 to 120 °C. Leaves of *E. longifolia* and *Citrus hystrix* were classified in the same group where they both exhibited a waxy leaf surface. Results also revealed that *E. longifolia* and *Pandanus amaryllifolius* shared the greatest similarity (ca. 80.7%).[44]

Lead content was assessed in 100 pharmaceutical dosage forms of Tongkat Ali products in Malaysian market (either registered or unregistered with the Drug Control Authority in Malaysia) using atomic absorption spectroscopy.[45] Eight products possessed 10.64−20.72 ppm of lead and, thus, did not comply with traditional medicines quality requirements in Malaysia. Even though only 92% of the products complied with quality requirements, they still cannot be assumed safe from lead contamination owing to batch-to-batch inconsistency.[45] In another study, the mercury content was analyzed in 100 products containing Tongkat Ali in various pharmaceutical dosage forms using cold vapor atomic absorption spectrophotometer. Mercury levels ranged between 0.52 to 5.30 ppm in 36% of the products and, thus, were not complying with Malaysian quality requirement for traditional medicines.[46]

5.3. Authentication and Adulteration of *E. longifolia* Herbal Products. High performance liquid chromatography (HPLC) offers several advantages for the authentication of natural products including advanced reproducibility in both identification of active constituents and their quantitative determinations. Besides, analysis is generally nondestructive and could be achieved in a relatively short period of time.[47,48] A study conducted by Abubakar et al. evaluated the extent of adulteration in *E. longifolia* herbal medicinal products via DNA barcoding followed by further validation using HPLC analysis.[49] Results revealed that 37% of the tested herbal medicinal products were authentic as revealed by DNA barcoding, whereas 27% were adulterated. The ITS2 barcode region proved to be the perfect marker. Meanwhile, HPLC analysis revealed a species, though it was identified by DNA barcoding as authentic; however, it did not contain the expected chemical compounds. It was suggested that DNA barcoding should be utilized by herbal industries as an initial screening step for raw material authentication prior to the manufacturing of herbal medicinal products. Further validation by chemical analyses would provide more information regarding their safety and efficacy.[49]

A highly sensitive fast technique for detection of adulterants in *E. longifolia* commercial products was conducted by Fadzil et al.[50] using high resolution melting (HRM) coupled with DNA barcoding (Bar-HRM). Both selected DNA markers possessed a distinguishable melting temperature. The HRM assay proved to be a reliable simple method for detection and identification of *E. longifolia* in herbal products. The DNA barcode combined with HRM not only recognized faked products from authentic ones, but also displayed sensitive detection levels.[50]

A multichannel sensor fabricated in-house incorporating an array of artificial lipid-polymer membrane was developed as a fingerprinting device for *E. longifolia*. A bioelectronic tongue that mimics the human gustatory system was used, and an artificial lipid material was incorporated as a sensing element.[51] The different plant parts, maturity stage, mode of extraction, and batch-to-batch variation of *E. longifolia* could be determined using this in-house fabricated multichannel sensor through the acquired potentiometric fingerprint and chemometric analysis. The incorporation of this sensor allows the quality control of herbal medicines, offering the advantages of being less time-consuming, less complicated, and unexpansive compared to conventional chromatographic techniques. However, quantification of marker compounds still need to be validated in complex herbal mixtures to fully assess the analytical capability of such sensors in nutraceuticals applications.[51]

Two-dimensional electrophoresis (2DE) was applied for positive detection of proteins in *E. longifolia* root aqueous extracts. Four spots could be observed. A pronounced Coomassie-stained spot was separated and subsequently referred to as Marker A. Chromatographic analysis of the aqueous extract led to the isolation of a pure protein from Marker A. 46 Products containing *E. longifolia* were randomly selected from markets worldwide and tested for Marker A. Twenty products were found to display results comparable to those obtained using eurycomanone as a marker. Products' ranking from the highest to the lowest quantity revealed a different order when compared for both markers. Marker A corresponded to its protein content and eurycomanone, a quassinoid, an organic molecule. Marker A detection via 2DE could be useful to test for the quality of *E. longifolia* root supplements.[52]

6. ANALYSIS OF *E. LONGIFOLIA* BIOACTIVES IN BIOFLUIDS FOR EFFICACY AND SAFETY PURPOSES

Interestingly, *E. longifolia* root extract has gained the attention of both scientists and athletes owing to its androgenic potential. It has been reported that *E. longifolia* improved spermatogenesis and fertility in male rats through the hypothalamic−pituitary−gonadal axis and hence results in increased testosterone production.[53] The testosterone-boosting effect of *E. longifolia* increases its potential for abuse in sports in terms of athletic performance, lower body fat, and strength training.[54,55] Therefore, *E. longifolia* crude extracts are widely used as dietary supplements, particularly for bodybuilding purposes.[55] However, limited impact could be traced to the urinary testosterone:epitestosterone ratio used for abuse detection of exogenous testosterone in doping routine analysis, after a 6-week administration of an *E. longifolia* extract in male athletes.[56] Thus, it was crucial to study the metabolism of a number of compounds isolated from *E. longifolia* root extract to identify the potential target metabolites for doping analysis.

Bräuer et al. investigated for the first time the *in vitro* metabolism of eight *E. longifolia* chemicals by using liver microsome incubation. 5,6-Dehydro-eurycomalactone, 9-hydroxycanthin-6-one, 9-methoxycanthin-6-one, and 11-dehydroklaieanone exhibited phase I biotransformation. In addition, 9-hydroxycanthin-6-one glucuronide was formed by *in vitro*

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glucuronidation via liver microsomes.[55] Metabolites observed after ingestion of a single dose of either *E. longifolia* root extract or 9-methoxycanthin-6-one by one male volunteer were comparable to those generated *in vitro*. For doping analysis, Bräuer et al. concluded that the proposed targets suitable for detection of *E. longifolia* consumption varied according to the matrix. The glucuronides of 9-methoxycanthin-6-one and its metabolite 9-hydroxycanthin-6-one are proposed for urine detection, whereas for serum detection the parent unconjugated compound 9-methoxycanthin-6-one and 9-hydroxycanthin-6-one glucuronide were proposed.

Low et al. developed a validated HPLC analysis of eurycomanone in rat plasma after oral and i.v. administration of *E. longifolia* extract for pharmacokinetic and bioavailability studies.[57] I.V. injection of 10 mg/kg *E. longifolia* extract containing 1.9 mg/kg of eurycomanone resulted in a relatively high plasma level of eurycomanone which declined rapidly to reach a zero level after 8 h. A mean elimination rate constant (k_e) of 0.8 h^{-1}, a biological half-life ($t_{1/2}$) of 1.00 h, a volume of distribution (V_d) of 0.68 L/kg and clearance (CL) of 0.39 L/h/kg were recorded. In contrast, oral administration of eurycomanone resulted in a C_{max} of 0.33 μg/mL and a T_{max} of 4.40 h. Despite the administration of an oral dose 5 times higher, the eurycomanone plasma level was much lower compared to the intravenous route indicating the poor oral bioavailability of eurycomanone.[57]

Ahmad et al.[58] studied the physicochemical characterization and pharmacokinetic (PK) attributes of eurycomanone via a series of experiments in rats and mice. Results revealed that eurycomanone is a highly polar compound exhibiting high stability at various pH values, in both plasma and in liver microsome. No major degradation product was observed in the gastrointestinal tract and blood plasma. Moreover, eurycomanone exhibited a low plasma protein binding capability. Despite these favorable properties, eurycomanone displayed low permeability which hinders its absorption and hence its bioavailability *in vivo*. These results highlight the necessity to use a low dose of eurycomanone in assays aimed at evaluating its efficacy. Besides, further evaluation of eurycomanone efficacy *in vivo* seems crucial.[58]

Ebrahimi et al.[59] conducted an NMR-based metabolomics approach to assess the effect of *E. longifolia* extracts with various quassinoid levels on rats' sperm count followed by subsequent examination of urinary metabolic changes following 48 days of *E. longifolia* treatment. Four groups of male Sprague−Dawley rats, 6 rats each, were administered water (control group), 125 mg/kg of *E. longifolia* water extract (TAW), 125 mg/kg of *E. longifolia* quassinoid-deprived extract (TAQP), and 21 mg/kg of *E. longifolia* quassinoid-rich extract (TAQR). Urine samples were analyzed using NMR after 48 days, then animals were sacrificed for sperm count analysis. Results revealed that the sperm count was significantly higher in TAW and TAQR-treated groups compared to control and TAQP-treated groups. Urine ^{1}H NMR profiles displayed higher levels of benzoic acid, trigonelline, and alanine in the high-sperm count group. On the other hand, ethanol was at a higher level in the normal sperm count group suggesting that quassinoids are effective with regard to sperm count increase. Moreover, this study provided potential urine biomarkers suitable for quantitative analysis of sperm profile and the status of male fertility,[59] which has yet to be examined in humans.

Faisal et al.[60] investigated the effects of *E. longifolia* root extract on serum leptin in male rats in relation to its well-known effects on testosterone levels. Leptin, a 16 kDa protein hormone, plays a key role in the regulation of energy intake and expenditure, being involved in appetite and metabolism.[61] Studies reported that hypogonadal men with very low testosterone levels showed high leptin levels that significantly decline upon exogenous testosterone administration inferring that testosterone acts a modulator of leptin.[62] Likewise, Faisal et al.[60] revealed that *E. longifolia* root extract significantly reduced serum leptin level concurrent with an increase in testosterone levels. Eurycomanone, the major quassinoid in *E. longifolia* extracts, accounts to a large extent for *E. longifolia* health effects.[63] Hence, pharmacological or toxicological actions of *E. longifolia* could be proven by tracing eurycomanone levels in biological samples such as plasma. Rehman et al.[63] developed and validated a simple, rapid, sensitive, and reproducible hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC-LC−MS/MS) method to determine eurycomanone levels in rat plasma, which was further applied to carry out a pharmacokinetic study of eurycomanone in rat plasma following oral dosing. HILIC chromatography is suitable for the bioanalysis of hydrophilic and polar analytes, found likewise successful to the pharmacokinetic study of eurycomanone in rat plasma following oral administration of *E. longifolia* extract and the pure compound. The C_{max} and AUC_{0-t} were at 9.9 ng/mL and 37.1 ng h/mL for *E. longifolia* extract (2 mg/kg as eurycomanone) versus 40.4 ng/mL and 161.1 ng h/mL, for 10 mg/kg eurycomanone, respectively, with the potential to be used for pharmacokinetic studies, investigating the safety and efficacy of *E. longifolia* in humans.[63]

7. CONCLUSIONS

E. longifolia is an important traditional medicinal plant with a variety of health benefits with the roots being the most used. This review capitalizes on the novel trends made toward the production of *E. longifolia* bioactives using biotechnology along with extraction optimization methods for best yields and recovery. In addition, novel extraction methods of *E. longifolia* bioactives were described. Strategies to optimize the production of *E. longifolia* extracts, maximizing the yield and minimizing major losses, were reviewed to improve the inclusion of that major drug in nutraceuticals. Based on the established literature of *E. longifolia*, it is of value to emphasize the identification of its active constituents and cost-effective analytical approaches for QC assessment of its plant material and supplements. Besides, investigation of metabolism of its bioactives by using *in vitro* and *in vivo* models for detection of *E. longifolia* biotransformed metabolites in body fluids is needed to determine its pharmacokinetics and ensure its safety and efficacy. Results revealed that the major quassinoid in *E. longifolia* (eurycomanone) exhibits high stability at various pH values, in both plasma and liver microsomes, found to effectively reduce serum leptin levels concurrent with an increase in testosterone. Such a biological effect likely mediates enhanced muscle mass and strength for bodybuilding, though the exact action mechanism(s) is not well-defined. To fully elucidate health benefits of *E. longifolia*, further research on the mechanism(s) and effects of *E. longifolia* must be conducted aided by improved analytics for detection of its biotransformed metabolites inside the body.

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M.A.F., A.O.A. and I.M.A. wrote the main manuscript text. M.A.F. and I.M.A. prepared the figures. M.A.F., M.T., K.W., and I.M.A. reviewed the manuscript.

Notes

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