Proceedings of ICBioMed 2024 Workshop: Computational Proteomics in Drug Discovery and Development from Medicinal Plants
DOI: 10.54254/2753-8818/77/2024.LA19832

Thearubigins: a comprehensive study from formation mechanism to biological activity and application prospects

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Abstract. Thearubigins (TRs) are a class of water-soluble, acidic phenolic compounds that are generated through oxidative polymerization reactions during the fermentation of black tea. As the predominant constituents in black tea, TRs significantly influence its unique color, texture, and potential health benefits. However, compared to polyphenols in black tea with lower molecular weights, research on TRs is more limited due to their diverse and complex nature. This paper reviews the current status of research on TRs production, the progress in isolation and preparation methods, and discusses the pharmacological effects of TRs, including their roles in antioxidant, anti-aging, anti-mutagenic, anti-cancer, and anti-tumor activities, as well as their anti-inflammatory properties and potential in preventing obesity. It also explores the prospects of TRs' application in the fields of food coloring, chemical, and healthcare industries. Despite their potential, the isolation and purification of TRs remain challenging due to their structural complexity and heterogeneity. Future studies should integrate various scientific techniques to further explore the bioactivity and mechanisms of action of TRs. The aim of this study is to provide a comprehensive overview that will serve as a reference for future research and application of high molecular weight polyphenols, such as TRs.

Keywords: Thearubigins, tea polyphenols, function, isolation.

1. Introduction

Since ancient times, tea has been an indispensable beverage in people's daily life. Presently, tea has attracted considerable consumer interest, ranking it as the runner-up in popularity following water, with black tea being the predominant choice, accounting for 78% of total tea consumption [1]. Furthermore, the prospective health advantages of black tea have consistently earned it high regard among scholars [2,3]. Making black tea involves four key steps: withering, crushing, fermentation and drying, of which fermentation is the most important step [4]. In the fermentation phase, tea leaves' polyphenols experience oxidative polymerization, driven by polyphenol oxidase (PPO) and peroxidase (POD), leading to the creation of theaflavins (TFs) and thearubigins (TRs) [1]. This process gives tea its characteristic reddish-brown colour.

Lately, the study of low molecular weight polyphenols in black tea has intensified, whereas investigations into higher molecular weight TRs, present in a significantly larger fraction (15-20%) in black tea, remain in the Roberts' phase. Despite the lack of agreement on the present interpretation of TRs, it's widely acknowledged that they consist of diverse phenolic polymers created through the polymerization of catechins and TFs. Due to the heterogeneity of TRs, their isolation and purification

DOI: 10.54254/2753-8818/77/2024.LA19832

remain challenging, leading to the fact that their associated chemical structures, specific compositions and properties are still unknown and need to be further explored in the future [1,5]. Current pharmacological research on TRs reveals their diverse biological functions, including antioxidant, antimutagenic, anticancer, anti-teratogenic, and obesity prevention effects, garnering interest in the medical and healthcare sectors [5]. In addition, despite the stability and cost advantages of synthetic colours, the potential safety hazards of synthetic colours have led to the replacement of natural food colours as an inevitable trend. These factors suggest that TRs hold great potential for future product development and application.

This paper reviews the progress of research on the formation mechanism, isolation method, biological efficacy and application of TRs in recent years. It expects that by summarising and analysing the relevant literature, it can provide a comprehensive empirical study of TRs and a reference for the subsequent research and application of high molecular weight polyphenols.

2. Pathway of TRs' formation

2.1. Basic properties and classification of TRs

Thearubigins are a class of water-soluble, acidic, reddish or maroon phenolic compounds with molecular weights ranging from about 700-40,000 Da, constituting 15-20% of black tea's dry mass and roughly 70% of its polyphenols [1,4,6].

There are two main ways of classifying TRs. The first is based on solubility, which Roberts classifies into three categories: water-soluble TRs SII, ethyl acetate-soluble TRs SI, and TRs SIa, which is soluble in water and ether [7,8]. The second method is to classify them according to their chromatographic behaviour in reverse high-performance liquid chromatography (HPLC) into three categories: firstly, non-column adsorbed TRs, secondly, soluble TRs with detectable discernible peaks, and thirdly, insoluble TRs showing "Gaussian hump" type insoluble peaks [4,9].

2.2. Mechanism of formation of TRs during black tea fermentation

The formation of TRs is achieved through the catalysing and oxidative reactions of several enzymes during black tea fermentation. Haslam's study showed that the main roles involved are polyphenol oxidase (PPO) and peroxidase (POD) [6]. As early as Roberts's study, it was suggested that TRs may be produced from epigallocatechin and its esters through a series of reactions in which TFs may be an intermediate product [8]. In 2010, Kuhnert used LC-MS to characterise the structure of TRs and proposed a new formation mechanism, the oxidative cascade hypothesis [10]. In addition, Yassin used mass spectrometry to monitor the electrochemical oxidation reaction of flavan-3-ol substrates in a study modelling the mechanism of TRs' formation and found that the oxidation occurs mainly at the B-ring and galloyl groups, generating TFs, catechin dimers and polyhydroxy flavan-3-ol derivatives [11]. Although the exact reaction conditions for TRs' formation are not clear, PPO, POD and oxygen are essential factors, while the enzymes involved in the reaction process require suitable pH and temperature. Studies have shown that the reaction time has a greater influence on the formation of TRs than temperature and that a longer oxidation time contributes to the degradation of TFs and the production of TRs [12,13].

3. Extraction and preparation of TRs

3.1. Organic solvent extraction

TRs are a complex pigment composed of several heterogeneous phenolic compounds, and their separation and purification processes are extremely challenging [5]. The extraction and purification of TRs are a challenging process, and with the continuous innovation of separation technology, various emerging methods have been introduced into the field of extraction and purification of TRs, but most of these methods are based on the traditional organic solvent extraction and chromatographic separation methods.

DOI: 10.54254/2753-8818/77/2024.LA19832

Roberts initially employed liquid-liquid extraction with organic solvents (employing ethyl acetate and n-butanol) for the isolation and characterization of TRs [7]. The traditional method of isolating and defining TRs was applied for the first time. The process begins with the preparation of tea broth by mixing black tea with boiling water, followed by extraction by successive application of chloroform and ethyl acetate to remove caffeine that may interfere with subsequent steps [14]. Subsequently, the ethyl acetate extract is dehydrated through evaporation, the remaining substance is solubilized using acetone, and a gradual precipitation process involves adding chloroform and ether to eliminate contaminants and separate the TRs segments, each with varying solubilities. Ultimately, the precipitates were eluted and purified by extracting with n-butanol and adjusting the solvent polarity to obtain a purer TRs fraction.

During the 1960s, Brown expanded upon Roberts' research by employing ethyl acetate, n-butanol, and acidic n-butanol for tea broth extraction and decontamination, successfully creating five distinct TRs fractions: TR-1 (dissolvable in ethyl acetate and acetone, insoluble in ethyl ether), TR-2 (soluble in n-butanol and methanol, insoluble in ethyl ether), TR-3 (soluble in n-butanol and acetone, insoluble in ethyl ether), TR-4 (soluble in acidic n-butanol and methanol, insoluble in ether), TR-5 (soluble in acidic n-butanol and acetone, insoluble in ether) [14]. By 2006, Krishnan et al. improved on the method of Brown et al. by using Soxhlet sequential extraction to extract five TRs (PBP-1 to PBP-5) from black tea broth that were free of catechins, TRs, and caffeine, which means that these fractions did not contain other bioactive monomers and oligomers [15].

3.2. chromatographic separation

Roberts' work laid the groundwork for the isolation and purification of TRs and became a key starting point for the field. Nevertheless, due to the complexity and heterogeneity of the TRs themselves, the TRs obtained by Roberts' method were not completely pure, but rather a series of mixtures with similar solubility and chromatographic properties. As chromatographic methods evolve, various enhanced separation methods have been utilized in the purification of TRs, encompassing high-speed countercurrent chromatography (HSCCC), HPLC, and Sephadex LH-20 column chromatography.

Cattell pioneered the initial hierarchical segregation of TRs in 1976 through Sephadex LH-20 column chromatography, effectively dividing the TRs into three segments according to their molecular weight and polarity variances, utilizing a 60% acetone (v/v) wash method [16,17]. Sephadex LH-20 column chromatography continues to be a prevalent technique for the chromatographic segregation of TRs to the present. McDowell I and colleagues in 1995 utilizing the HPLC method, 38 phenolic peaks were extracted from black tea broth, with 22 recognized as TRs, and it was discovered that TR2 and TR12 had a strong correlation with the quality of black tea [18]. Since the SI fractions isolated from the TRs usually contain more catechins and TFs, the current study focused more on the TRs SII fractions [19].

Due to the effectiveness of High-Speed Countercurrent Chromatography (HSCCC) in separating finer particles, Degenhardt and other researchers adopted this technique to obtain a better quality TRs SII fraction. In the year 2000, Degenhardt achieved a more refined TRs fraction through the combination of tea broth extract and ethyl acetate-n-butanol-water (2:3:5) solvent, followed by its separation post-treatment with the ion exchange resin XAD-7 [20].

4. Biological effects of TRs

4.1. Antioxidant and anti-ageing effects

Research has consistently focused on the link between tea consumption and health, particularly about conditions like high blood pressure, elevated blood sugar, high blood fat, and other three major population symptoms, where moderate tea intake has shown substantial health advantages. Green tea contains high levels of catechins, which are essential for its numerous health benefits. Black tea contains more TRs than green tea, which has led researchers to explore the link between TRs and health benefits. The presence of the phenolic hydroxyl group alongside the benzophenone framework in TRs contributes to their pronounced biological activity, which is crucial for their pharmacological properties. Research highlights that TRs exhibit considerable capabilities in neutralizing free radicals and displaying

DOI: 10.54254/2753-8818/77/2024.LA19832

antioxidant properties. Additionally, these compounds have demonstrated notable advantages in cancer prevention, the reduction of mutagenic effects, inhibition of toxins, and enhancement of gut health [5].

In numerous chemical modelling experiments, TRs have demonstrated antioxidant properties similar to those of catechins and TFs. In 1994, Yoshino conducted a chemical experiment using tert-butyl hydroperoxide (t-BHP) to trigger lipid peroxidation in rat liver. The study revealed that TRs could demonstrate their antioxidant properties by modulating the activity of specific biological enzymes and directly neutralizing free radicals, thereby effectively countering lipid peroxidation [21]. In addition, the anti-lipid oxidation effect of TRs even exceeded that of glutathione, L(+)-ascorbic acid, dl-α-tocopherol, 2,6-di-tert-butyl-4-methylphenol (BHT), and butylated hydroxyanisole (BHA). According to the research conducted by ILuczaj et al., the theaflavins present in black tea have demonstrated antioxidant properties by preventing the formation of free radicals, neutralizing existing free radicals, and binding with transition metal ions [22]. Meanwhile, TRs can regulate transcription factors like NF-κB and AP-1, and although the antioxidant efficacy of TRs is slightly lower than that of EGCG and TFs, its role in the regulation of cellular signalling is still of great value. Imran in a 2018 study, dietary intervention with TRs was found to significantly improve lipids, glucose levels, renal function, and thiobarbituric acid reactive substances (TBARS), as well as enhance insulin, high-density lipoprotein (HDL), and haematological parameters in arginine-induced renal dysfunction rats [23]. Some studies also showed that the antioxidant capacity of TRs was superior to common antioxidants such as ascorbic acid, glutathione as well as tocopherols, but still lower than that of catechins and TFs [24,25].

TRs also have anti-ageing potential. Cells are the basic units that make up human tissues, and cellular ageing is closely related to the ageing of the whole organism. In experiments on the protective effects of oxidative damage in HPF-1 cells, TRs showed a strong antioxidant capacity, which contributes to anti-ageing. Reactive oxygen groups or molecules (ROS: O^{2-}, OH^{-}, H_2O_2) produced during human metabolism can cause the accumulation of oxidative damage, limiting vital metabolic activities and ultimately leading to ageing. Recent in vitro experiments have demonstrated the ability of TRs to scavenge ROS and reported that TRs can protect against DNA breaks induced by oxidative damage by scavenging ROS.

4.2. Anti-cancer, anti-mutagenic and anti-teratogenic effects

Numerous studies have shown a correlation between black tea consumption and a reduced incidence of several cancers [26]. Specifically, TRs enhance the inhibitory effects of certain substances on the growth of cancer cells. Sakamoto's study found that TRs alone did not have a significant effect on prostate tumour cells (PC-3), but when combined with trihydroxyisoflavones, there was a good synergistic effect, which was able to effectively inhibit the proliferation of prostate tumour cells [27].

In addition, Lodovici found that the ethyl acetate fraction of TRs was effective in preventing 1,2-dimethylhydrazine-induced DNA breaks in rat colonic mucosa, thereby enhancing immune function and reducing the risk of intestinal cancer [28]. Patela et al. further showed that TRs modulate the activity of antioxidant response element (ARE) in hepatocytes and enhance the stability of the NRF2 gene through the phosphatidylinositol-3-kinase (PI3K) pathway, which induces the synthesis of toxin-scavenging proteins and enzymes, and has a positive effect on anticancer [29]. Patela's team also pointed out that TRs were able to inhibit DMH-induced colonic rectal tumours, and that its protective effects may reduce tumour size and diversity through b-catenin regulation [30].

In addition, TRs enhance the expression of the oncogenic protein Bax, thereby increasing the ratio of Bax to Bcl-2, and up-regulates the expression of p19, p21, p27 and p53, inhibits the phosphorylation of the cell survival signalling protein AKT, which in turn enhances the anti-apoptotic ability of normal cells, induces the production of reactive oxygen species (ROS), and promotes the apoptosis of human skin cancer cells [31].

Current studies on human lymphocyte cultures, human epidermoid carcinoma cells, human malignant melanoma cells, human leukaemia cells (U9 37), human gastric carcinoma cells (MKN45), colon carcinoma cells, lung carcinoma cells, and Amish Salmonella assay on different Salmonella strains have demonstrated the anticancer and antimutagenic effects of TRs [4].

DOI: 10.54254/2753-8818/77/2024.LA19832

Beyond their roles in combating cancer and reducing mutagenicity, TRs also display anti-teratogenic properties. This is achieved through various mechanisms, including the suppression of promoter activation, the stimulation of DNA repair processes, and the detoxification of harmful mutagens and carcinogens [4]. Gupta and colleagues demonstrated that TRs effectively shielded Swiss mice from teratogenic effects induced by cyclophosphamide and dimethylbenzanthracene. Their assays of sister chromatid exchanges (SCE) and chromosomal aberrations (CA) revealed notably beneficial protective impacts [32]. In vitro experiments by Halder further demonstrated the protective effect of TRs against the inducers benzo(a)pyrene and aflatoxin B1, with protective rates of 20-52% and 16-56% in the chromosomal aberration (CA) assay and 19-43% and 16-56% in the micronucleus (MN) assay, respectively [33].

4.3. Antileukaemic, antitoxic and antiviral effects

Halder et al. group found that in the human leukaemia cell lines U-937 and K562, TFs and TRs induced cell arrest in the G0/G1 phase by inhibiting the overexpression of the AKT signalling pathway [34]. This process resulted in up-regulation of p19, p21 and p27 expression, along with down-regulation of the levels of CDK2, CDK4, CDK6 and CD1, thus acting as a chemopreventive agent against human leukaemia cells.

TRs have also shown inhibitory effects on toxins. Satoh et al. used n-butanol soluble TRs fraction in vitro experiments on mice and found that it had a significant inhibitory effect on botulinum toxin type A [35]. In addition, studies have shown that TRs can exert their effect against tetanus by binding to the tetanus toxin [36].

It is worth emphasising that TRs also have an important contribution to make in the fight against HIV. The findings of the research indicated that TRs effectively suppressed the functions of reverse transcriptase as well as both DNA and RNA polymerases associated with the HIV, albeit to different extents [5].

4.4. Anti-inflammatory improves digestive effects

2,4,6-trinitrobenzene sulfonic acid (TNBS) is a weakly acidic semi-antigen that does not induce an immune response when present alone. However, following ethanol damage to the intestinal mucosa, TNBS can be converted into a conjugated substance and induce an immune response, generating antigens. Upon antigenic stimulation, T cells are converted into sensitised lymphocytes, which in turn attack host cells bound to the semi-antigen, leading to inflammation in the gut. In the TNBS-induced inflammatory bowel disease model in mice, reactive oxygen species (e.g., O^{2-}, H_2O_2, etc.) and peroxides generated by the metabolism of TNBS damage intestinal wall tissues and epithelial cells. By scavenging these anionic ions, TRs alleviate the damage to the intestinal structures of the mice, significantly alleviate diarrhoea and reduce the level of lipid peroxidation. Maity and colleagues demonstrated that administering 40 mg/kg of TRs daily to mice proved beneficial in eliminating pathogens, minimizing damage to intestinal structures, and alleviating both diarrhoea and colonic inflammation [37].

Black tea TRs can also optimise the intestinal microbial environment of the human digestive system and maintain physiological balance, especially significant for the middle-aged and elderly groups. TFs and TRs notably inhibit intestinal bacteria and can boost immune function in the gut. Miyata et al. observed an increase in faecal steroid excretion and a decrease in cholesterol levels in mice fed phenolic substances such as TRs [38]. Murad et al. demonstrated that tea extracts significantly influenced intestinal microflora. Miyata et al. also showed that tea extracts BTE (4.5%) and TRs (6 mg/kg) were able to alleviate sildenafil-induced dyspepsia [39].

Liu and colleagues suggest that the way black tea polyphenols engage with the gut microbiota could play a role in managing gastrointestinal motility [40]. Research revealed that TRs enhanced delayed gastric emptying and small intestine function in mice, triggered by sildenafil, in a manner reliant on the dosage [39]. The mitigating impact might be somewhat hindered by L-NAME, known for its beneficial role in reducing dyspepsia.

DOI: 10.54254/2753-8818/77/2024.LA19832

4.5. Prevention of obesity

Excessive body fat buildup, particularly triglycerides, leads to obesity, a health disorder. 2008, Cameron and colleagues discovered that TRs and TFs produce effects akin to insulin in human cells, reducing blood sugar levels and enhancing the production of proteins, fats, and glycogen [41]. In addition, they activate the transcription factor FOXO1A, which is associated with longevity, through a phosphorylation process and inhibit the activity of the enol pyruvate phosphoryl carboxylate kinase (PEPCK), showing potential anti-obesity and anti-ageing effects.

Concurrently, Kusano and colleagues discovered that TRs' suppressive impact on lipase activity in n-butanol-extracted black tea in vitro paralleled that of ethyl acetate-extracted black tea extracts and TFs gallate, which were (46.8±5.4)%, (47.7±2.6)%, and (48.9±2.8)%, respectively, but this effect was significantly better than that of epigallocatechin gallate (EGCG), which was 14.5±13.2% [42]. The in vitro inhibitory effect of TRs on α-amylase was (57.0±1.8)%, which was lower than that of black tea ethyl acetate extract (65.4±4.1)% and TFs gallate (81.6±2.8)%. An in vitro study by Uchiyama et al. showed that the IC50 values for pancreatic lipase inhibition by black tea extracts BTPE and TRs were 15.5 and 36.4 μg/mL, respectively [43].

BTPE demonstrated the ability to lower plasma triglyceride levels in Wistar male rats on a high-fat diet, indicating a correlation with dosage. Conversely, in female C57BL/6N mice, BTPE succeeded in preventing the rise in body weight gain, peri-uterine fat tissue mass, and liver fat due to a high-fat diet. Li suggested that these effects may be related to the inhibition of small intestinal lipid absorption by BTPE, of which TRs are a major component [44]. Taken together, these findings lead to the inference that TRs may be effective in preventing obesity.

5. Application of TRs

5.1. food colouring

TRs, as a versatile pigment and preservative, have received research attention from scientists in several countries, including the former Soviet Union and the United Kingdom, who have concluded that tea pigments are excellent food colouring agents. Relevant studies have pointed out that dried beans untreated with any additives will undergo spoilage in just one day in a 30 ℃ environment. In contrast, dried beans with added TRs showed insignificant changes in colour and odour even after 4 days of storage at the same temperature, and the physical properties of the product such as elasticity, hardness, water retention and flavour were not adversely affected. Due to the high colour value and significant antioxidant capacity of TRs, they are considered as a class of high-quality functional food colours.

5.2. Chemical & Healthcare

Owing to their superior antioxidant properties and ability to neutralize free radicals, TRs are considered to have significant pharmacological effects in the prevention of cardiovascular and cerebrovascular diseases, anti-bacterial, anti-viral, and ageing, making them ideal raw materials in the chemical and healthcare fields. Guangzhou Poiseau Trading Co., Ltd. has launched the "Tea Doctor-Tea Red Vegetables Youth Fixing Set", which is now on the market, and is considered to be a high-quality cosmetic product due to its skincare, antioxidant and anti-ageing properties. Jiangxi Green Group produced the tea colour capsule after clinical trials, which has been proven to have a better therapeutic effect on cardiovascular and cerebrovascular diseases, and has been put into the market. With the in-depth research on the pharmacological effects of TRs and the continuous isolation and purification of their active ingredients, the new fields of TRs in clinical applications will be expanded.

6. Conclusion

TRs, a crucial pigment element in black tea, hold significant promise for practical use. The article sheds light on its various bioactive roles, assesses its possible health advantages, and offers perspectives on the prospective uses of TRs.

DOI: 10.54254/2753-8818/77/2024.LA19832

Despite evidence from both in vivo and in vitro research confirming TRs' diverse pharmacological roles, the extent to which TRs perform their anticipated biological functions in humans is yet to be determined in upcoming clinical trials.

Furthermore, segregating TRs segments remains difficult owing to the intricate and diverse nature of TRs configurations, leading to varied separation techniques in diverse research, often producing combinations of black tea extracts. Differences exist in the purity levels of the TRs examined, influencing the compelling outcomes of these studies. Currently, it appears that merging various techniques based on their intended use is the most efficient strategy.

Presently, the formation process of TRs lacks consensus, yet evidence suggests that TRs can originate from catechins oligomers via distinct polymerization processes. Utilizing diverse mass spectrometry methods offers a viable way to hypothesize about TR formation, yet it's equally crucial to identify more efficient approaches for TR research.

Future directions for TRs research may include the following:

1. Combining different separation methods or using enzymology, enzyme engineering and high-end analytical techniques for the isolation and purification of TRs to prepare purer fractions of TRs.
2. Develop environmentally friendly and efficient TRs extraction technology to reduce energy consumption and environmental pollution in the production process.
3. Bioactivity studies using modern pharmacological methods and clinical studies to confirm the possible biological activity of TRs in humans.
4. Utilize mass spectrometry or innovative techniques to explore the genesis of TRs, aiming to deepen our comprehension of TRs' formation and structure.

Generally, research prospects for TRs appear hopeful, yet there remains a significant journey ahead to completely comprehend and apply them. Upcoming studies should amalgamate diverse scientific methods to thoroughly investigate the biological functions and action mechanisms of TRs, aiming to harness their capabilities in enhancing human health.

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