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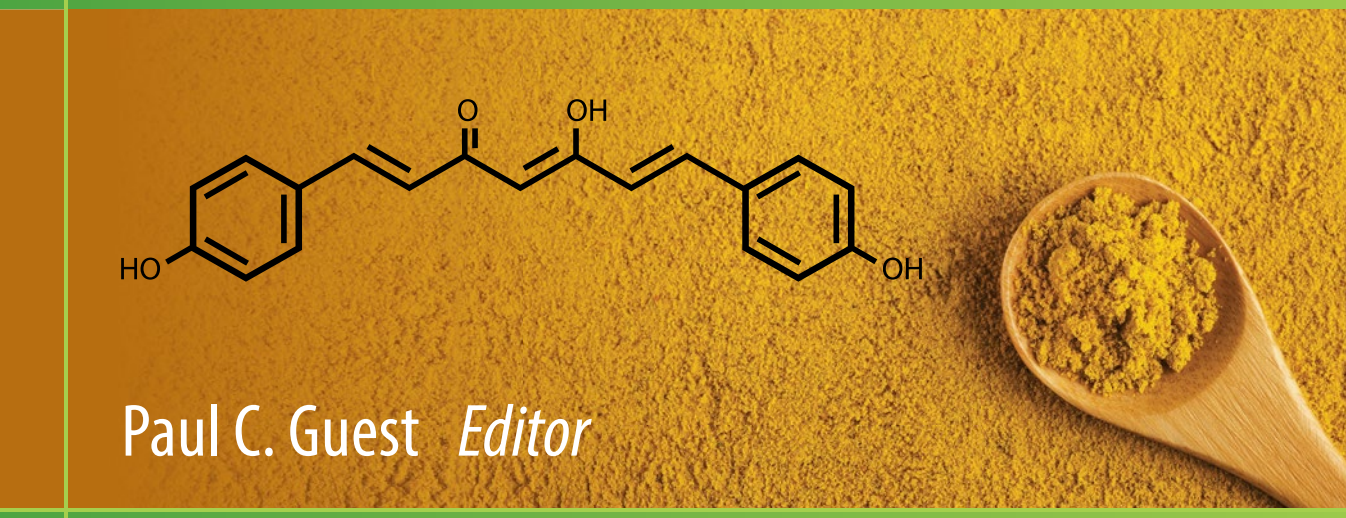

Paul C. Guest *Editor*

Physical Exercise and Natural and Synthetic Products in Health and Disease

Humana Press

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Edited by

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Charlesworth House, Debden, Essex, UK

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Preface

According to the World Health Organization, noncommunicable diseases impose the greatest burden on global health with staggering costs in daily associated living and to the healthcare services. Beyond traditional pharmaceutical approaches, thousands of studies have now been carried out with the aim of testing aerobic exercise, resistance training, special diets, additives, and natural products, which have led to new insights into the physiological and molecular aspects of health and disease. Many of these approaches have led to significant improvements in disease areas such as cardiovascular disease, cognitive dysfunction, diabetes, frailty, glioblastoma, metabolic syndrome, obesity, oxidative stress, and various cancers. This book presents a series of protocols covering such studies by research scientists and clinicians from five out of the six habitable continents. This includes countries such as Brazil, Canada, China, Germany, India, Indonesia, Iran, Oman, Philippines, Poland, South Africa, South Korea, Thailand, the United Kingdom, the United States of America, and Vietnam. This underscores the keen interest in the possibilities of natural remedies throughout the world.

The book will be of high interest to researchers in the areas of chronic disease, exercise, and nutrition, as well as to clinical scientists, physicians, and the major drug companies since it gives insights into possibilities for the development of novel therapeutics, as well as the means of monitoring therapeutic response through measurement of molecular and physiometric biomarkers. It will also be of high interest to both technical and bench scientists as it gives detailed instructions on how to carry out the various presented methods along with important notes which give insights beyond the traditional protocols. It also provides important information on disease mechanisms and novel drug targets as each protocol is presented in the context of specific chronic diseases or different therapeutic areas. Finally, it will be of high interest to people in all walks of life considering that physical health has been linked to disease outcomes in the current COVID-19 pandemic.

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Part I

Reviews

Chapter 21

Assessment of Topical and Transdermal Penetration of *Curcuma heyneana* Rhizome Extract in Rat Skin: Histological Analysis

Idha Kusumawati, Rohmania, Mega Ferdina Warsito, and Eka Pramyrtha Hestianah

Abstract

Currently, there are increasing numbers of dermal and transdermal dosage forms of both natural and synthetic compounds on the market. Therefore, it is necessary to have a method that can measure the release and penetration of the compound into the skin. This chapter presents a current method for evaluating the skin penetration of a *Curcuma heyneana* rhizome extract in vivo using histological parameters. We also evaluate a liposome delivery system of the same extract to determine any differences in penetration due to changes in the drug delivery system.

Keywords Skin penetration, Skin permeation, *Curcuma heyneana* rhizome extract, Liposomes, Histological analysis

1 Introduction

Recently, research into the development of transdermal and dermal dosage forms of natural therapeutic agents has increased. This skin-applied dosage form can deliver a minimal or noninvasive therapeutic agent into the body, which has an advantage over other delivery routes. This is in line with the increasing development of new drug delivery systems [1, 2]. The development of topical and transdermal drug delivery systems shows a significant advantage in effectively hitting drug targets in the body, thereby reducing systemic side effects. Also, such approaches can be an alternative to overcome the problem of oral drugs with low absorption and the occurrence of first-pass metabolic effects [1, 3].

For dermal and transdermal dosage forms, studies on drug penetration into the skin is a concern to determine the ability of drugs to reach their therapeutic targets. The penetration ability of a

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therapeutic agent is influenced by several factors such as the release of the agent from the carrier, the penetration of the agent through the stratum corneum and other skin layers, and the activity of the agent at the target point [1, 4]. In both dermal and transdermal dosage forms, the skin is a challenging barrier for efficient penetration of the therapeutic agent. The skin consists of three main layers, namely the epidermis, dermis, and hypodermis, and its thickness is influenced by body area, age, and gender [1, 2, 5, 6]. The factors affecting the release and penetration of the drug are the interactions between the drug, the skin, and the carrier.

The release and penetration of the drug from the dermal and transdermal dosage forms to the systemic circulation is a series of multistep processes which includes: (a) release from the preparation; (b) partition into the stratum corneum; (c) diffusion into the stratum corneum; (d) partition of the stratum corneum into the epidermal layer; (e) diffusion across the epidermal layer into the dermis; (f) absorption by blood vessels; and (g) reaching the systemic circulation [1].

In previous research, we have shown that the ethanol extract of *Curcuma heyneana* rhizome has an antioxidant and antiaging activity that protects the skin from the damaging effects of excessive UV exposure [7–9]. This study used a histomorphometric method of rats exposed to UV light [10]. The development of a drug delivery system is needed to bring drugs into the skin, subcutaneous tissue, and even to the systemic body. Herbal extracts contain a variety of complex chemical constituents that have properties from lipophilic to hydrophilic and have small to large chemical structures. This will affect the ability of these chemical substances to penetrate into the skin [7, 9]. To increase penetration, an ethanol extract of *Curcuma heyneana* can be made in the form of a liposome. In this chapter, we use a rat skin tissue layer model and describe an in vivo bioassay protocol for enabling the penetration of the ethanol extract of *Curcuma heyneana* rhizome labeled with fluorescent rhodamine.

2 Materials

2.1 Animals, Solutions, and Reagents

1. 200–300 g healthy adult male rats (2–3 months old) (*see* **Note 1**).
2. 1% rhodamine.
3. Standard rat chow.
4. 70% ethanol.
5. Water.
6. Ethanolic extract of *Curcuma heyneana* rhizome [7] (*see* **Note 2**).
7. Gel base (*see* **Note 3**).

8. Propylene glycol.
9. Phospholipon 90.
10. 100 mM phosphate buffer (pH 6.4) (*see* **Note 4**).

2.2 Equipment and Software

1. Electric razor.
2. Scissors.
3. Microdissection forceps.
4. Dissecting boards and pins.
5. Cryotome (Leica).
6. Microwave oven.
7. ULTRA-TURRAX® high-performance dispersing instrument (IKA; Staufen, Germany).
8. Rotary evaporator.
9. Inverted system microscope IX71-IX2 series optical microscope (Olympus; Shinjuku-ku, Tokyo, Japan) (*see* **Note 5**).
10. DP71 camera (Olympus).
11. Cell D software (Olympus).
12. Statistical analysis package (*see* **Note 6**).

3 Methods

3.1 Animals

1. Acclimatize rats for at least 5 days before use (*see* **Note 7**).
2. Set the temperature of the animal room at 22 °C (±3 °C) and the relative humidity at 30–70%.
3. Set the lighting cycle at 12 h light and 12 h dark.
4. Provide a conventional diet with access to water ad libitum.
5. Shave the back of each rat using the electric razor to expose a 3 × 3 cm area (Fig. 1).

3.2 Preparation of Curcuma heyneana Rhizome Extract

1. Wash the *Curcuma heyneana* rhizome, cut, and dry in an oven set at 40 °C for 3 days.
2. Grind the dried material (100 g) into powder.
3. Extract using 10 volumes of 70% ethanol by heating in a microwave at 30% power for 1 min.
4. Combine the extract with the 1% rhodamine solution.
5. Dry by evaporating the ethanol under reduced pressure to obtain a crude extract.

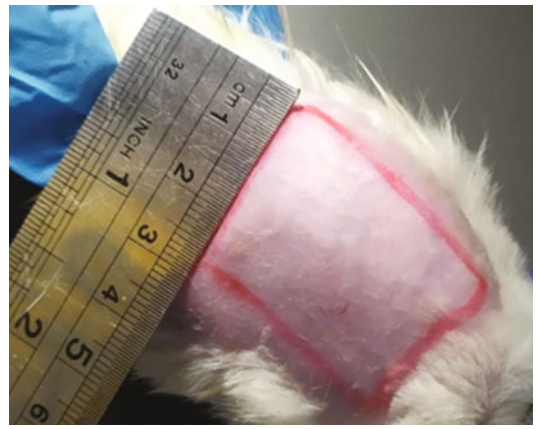

Fig. 1 Shaved back of the rat

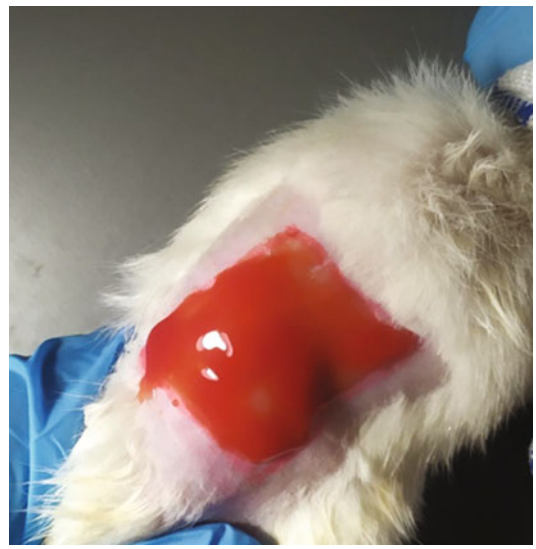

Fig. 2 Sample application on skin back of the rat

3.3 Preparation of Liposomes of Curcuma heyneana *Rhizome Extract*

1. Dissolve the Phospholipon 90 using 6 mL propylene glycol.
2. Mix the extract of *Curcuma heyneana* rhizome (containing 3 mg curcuminoids) into the Phospholipon 90 solution.
3. Stir the mixture using the ULTRA-TURRAX at a speed of 8600 rpm for 5 min.
4. Add 4 mL of the phosphate buffer slowly during stirring.

3.4 Treatment

1. Assign the rats randomly into three groups: vehicle (gel base), *Curcuma heyneana* rhizome extract in gel base, liposome of *Curcuma heyneana* rhizome extract in gel base.
2. Apply the sample (50 mg) topically to the shaved area.

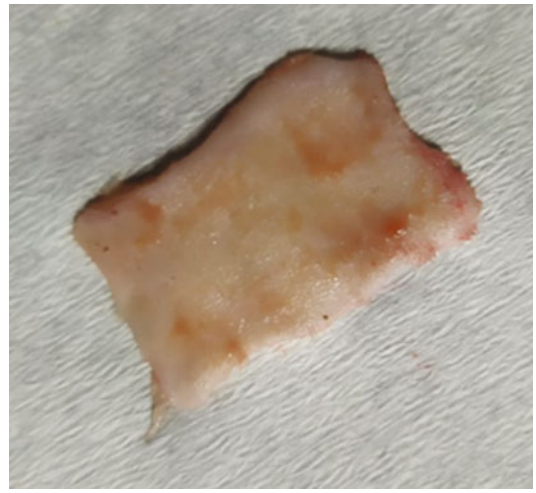

Fig. 3 Skin biopsy from the back of the rat

3.5 Biopsy and Histology Analysis

1. After 60 min, cull rats by cervical dislocation.
2. Remove the skin wounds from the shaved area using the dissection tools (Fig. 3) (*see* **Note 8**).
3. Freeze the skin biopsies using liquid nitrogen for fresh sectioning (2 μm) using a cryotome (Fig. 4) (*see* **Note 9**).
4. Observe and record images using the fluorescence microscope and digital camera (Fig. 5 and Table 1) (*see* **Note 10**).

3.6 Statistical Analysis

1. Present the results as mean ± standard error of the mean (SEM) and estimate statistical differences between groups using one-way analysis of variance (ANOVA) with Duncan's test, considering $p < 0.05$ as statistically significant.
2. Determine the ratio data of the scoring of the sample penetration into the rat skin layer (Table 1 and Fig. 6) (*see* **Note 11**).

4 Notes

1. Before starting the research, researchers should ensure that all procedures adhere to ethical standards for animal use and are approved by the appropriate institutional authority. This study was approved by the Animal Experiment Ethics Committee of Airlangga University (protocol number 1146/10) [7].
2. The ethanol extract of *Curcuma heyneana* rhizome used in this study was derived from previous research. *Curcuma heyneana* is a Zingiberaceous plant native to Java Island, Indonesia, known locally as Temu Giring, and is used for beauty treatments in Javanese and Balinese traditions [7].

Fig. 4 Histology slide showing fresh biopsy rat skin

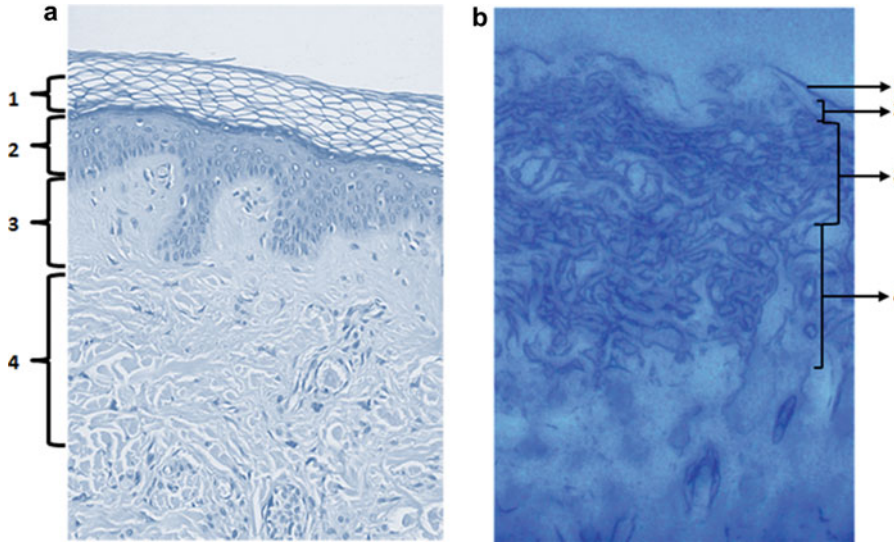

Fig. 5 (**a**) Diagram showing the basic layers of skin. (**b**) Experimental image showing scoring of the rat skin layers: (1) upper epidermis to stratum granulosum; (2) stratum spinosum to basal stratum; (3) upper dermis; and (4) lower dermis

Table 1
Criteria for scoring histological specimens

Score	Description
1	The sample penetration reaches the stratum corneum and the stratum granulosum layer
2	The sample penetration reaches the stratum spinosum and the stratum basal layer
3	The sample penetration reaches the papillary dermis
4	The sample penetration reaches the reticular dermis

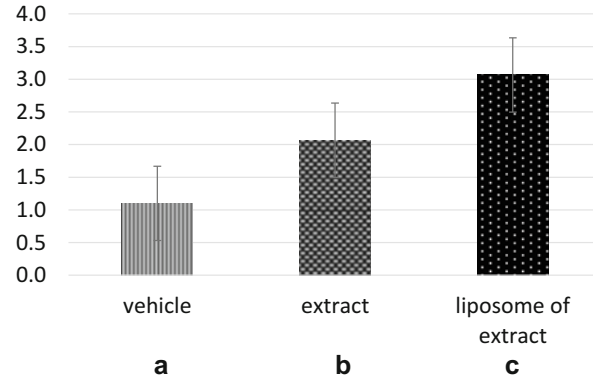

Fig. 6 Scoring of histologic analysis of drug penetration through rat skin. Values are expressed as mean ± SEM ($n = 6$). Means associated with each set of data are significantly different at $p < 0.05$ (Duncan's test) from either (a) vehicle (gel base) group, (b) *Curcuma heyneana* rhizome extract in gel base or (c) liposome of *Curcuma heyneana* rhizome extract in gel base

3. The vehicle used should be semisolid (cream- or gel-based) instead of liquid using a gel base. Attempts should be made so that this is similar to the *Curcuma heyneana* and retinoic acid samples. Both *Curcuma heyneana* extract and its liposomes are mixed into the gel base [7].
4. Many buffers can be used. Here, we use phosphate buffer pH 6.8 for liposome preparation.
5. In this study, we used an Olympus fluorescence microscope, camera, and analysis package. Other similar systems can be used although the user should ensure compatibility with the experimental procedures.
6. Several statistical software packages can be used such as Microsoft Excel (Redmond, WA, USA) and SPSS (SPSS Inc., Chicago, IL, USA).
7. Acclimatizing the rats and ensuring conditions are constant and otherwise comfortable can reduce stress for more reproducible results.

8. The rat skin biopsy should be immediately placed on filter paper to prevent it from creasing.
9. Histological slides should be analyzed immediately to prevent rhodamine fluorescence from fading.
10. The evaluation of liposome delivery system of the extract and its liposome can determine using the differences in penetration due to changes in the drug delivery system. The penetration of the sample was analyzed using rhodamine as an indicator [11–13]. The skin penetration level of the sample was determined semiquantitatively based on scoring system (Table 1 and Fig. 5).
11. According to the penetration test results, the extract could penetrate the upper dermis and the basal stratum. The presence of phospholipids in liposomes that mimic the skin membrane explains this.

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SCOPE

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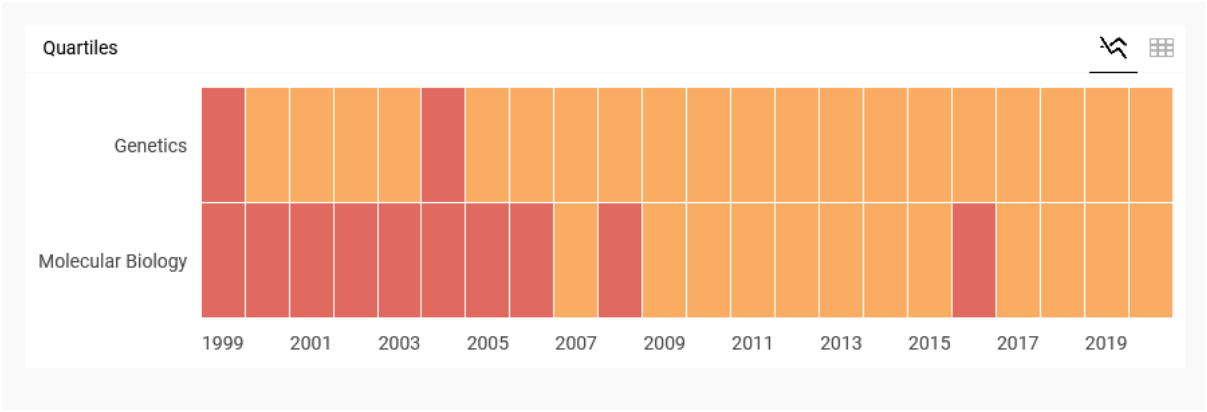
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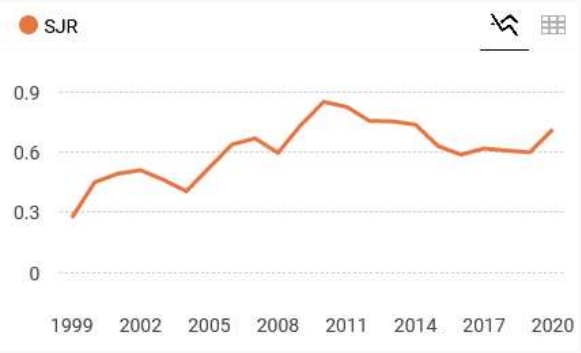

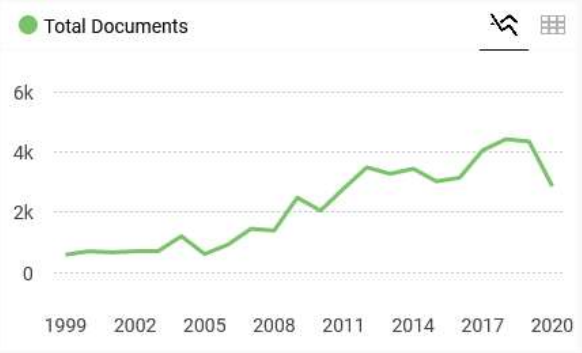

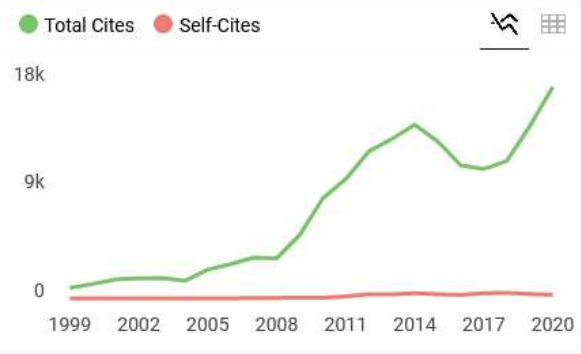

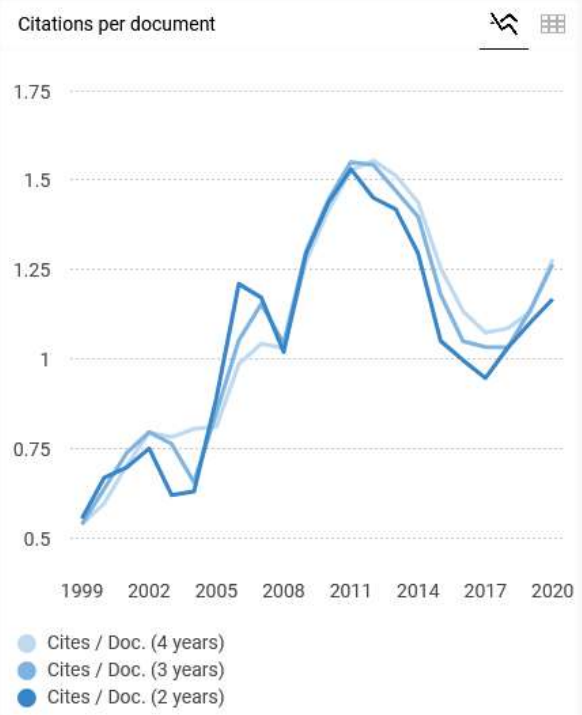

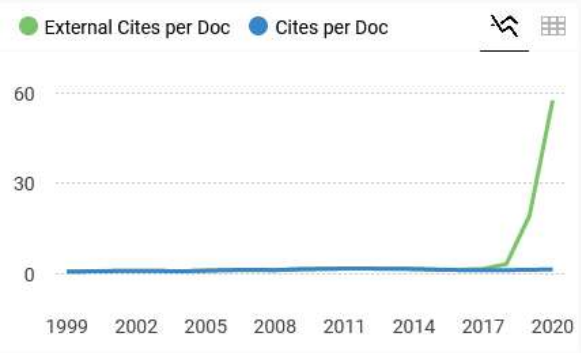

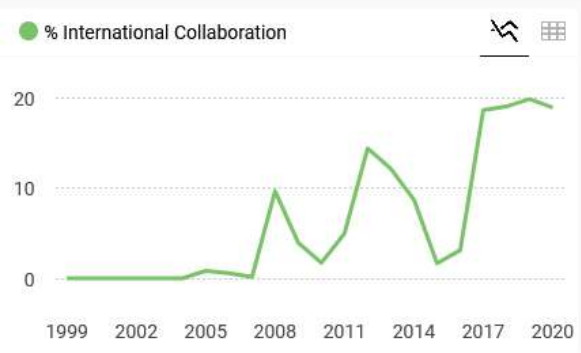

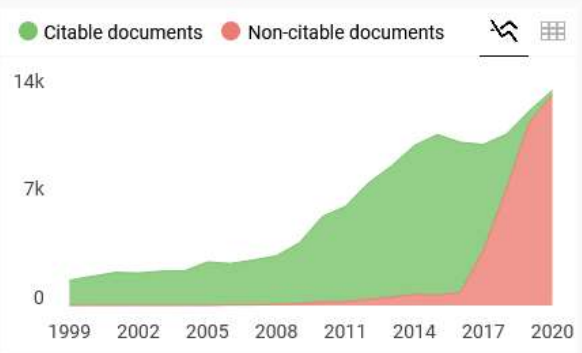

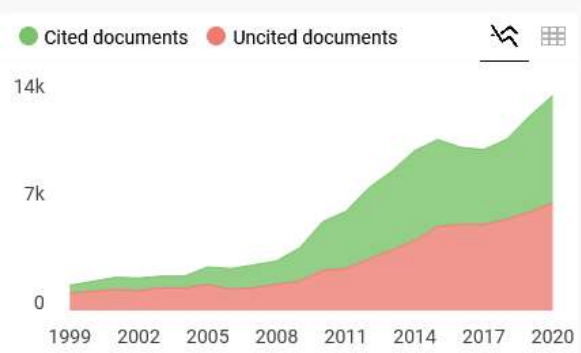

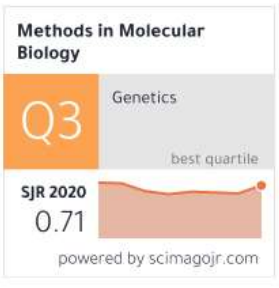

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