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Controllable Transdermal Drug Delivery of *Theobroma cacao* Extract Based Polymeric Hydrogel against Dermal Microbial and Oxidative Damage

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Abstract

Human dermal layers are directly bared to the external environmental adversities like pollution, radiation, dust along with various chemical and mechanical stress conditions which constantly lead to the oxidative stress, eventually forming free radicals. These conditions also support the dermal microbial infections by invading the cutaneous layers. Therefore, the most efficient approach accepted globally to combat these complications is to opt for transdermal application of exogenous antioxidants which helps in reducing the ill effects of oxidative stress and promotes the DNA repair. However, many scientific findings exhibited the potential role of *Theobroma cacao* for providing efficient skin protection. *Theobroma cacao* known for its vital procyanidin flavonoids, phenolic compounds, methylxanthines, catechin and epicatechin phytoconstituents with numerous health benefits, besides helping in smoothening and softening the damaged skin. These therapeutic benefits of cocoa phytocompounds are attributed to its anti-inflammatory and antioxidative characteristics. This study addresses the fabrication and optimizations of *Theobroma cacao* extract loaded carbopol hydrogel system for increased antioxidative and antimicrobial effects. It was found in the studies that the hydrogel-based *T. cacao* extract (TCHG) has significantly improved the therapeutic index of the extract, making it more suitable for transdermal application. The optimized hydrogel was further characterized by physicochemical parameters, SEM analysis and *in-vitro* release kinetics. The reported findings showed the sustained compound release with comparatively significant antimicrobial activity of TCHG against microbial (*Pseudomonas*

fluorescens, *Bacillus licheniformis*, *Micrococcus luteus*, *Aspergillus niger*, *Trichoderma harzianum*, *Rhizopus oryzae*) infections. Also, the higher ROS quenching ability of TCHG as compared to *T. cacao* extract promises to be an effective transdermal formulation.

Keywords

Theobromine, Hydrogel, Oxidative Stress, Release Kinetics, Transdermal

1. Introduction

Human skin is the most contacted and directly exposed organ known and thus, has multi-layered defense barrier systems for protection from external stress causes [1] [2] [3]. The dermal endogenous mechanism secures the cellular continuity, immunological balances, enzymatic defenses and cutaneous homeostasis, however, the exogenous compounds like ascorbic acid (AA), vitamin E, lycopene, cocoa etc. counter the quenching of ROS (Reactive Oxygen Species) besides, enhancing the antimicrobial properties [4] [5]. The flavonol and phenolic content of cocoa are also reported to contribute to enhancing the endogenous mechanism [6]. Cocoa also known as *Theobroma cacao* (*T. cacao*), sub-classified as Criollo, Forestero or Trinitario varieties has an essential phytoconstituents, theobromine (3,7-dihydro-3,7-dimethyl-1H-purine-2,6-dione) which belongs to the category of methylxanthines and affiliates to alkaloids (purine), affecting many physiological processes in body [7] [8] [9]. The pharmacological and toxicological properties of theobromine and other methylxanthines have been reviewed by many researchers and the chemical profile of roasted cocoa beans was found to be more complex [10]. But the primary compounds that induce its multiple beneficial functions are either naturally occurring or process-derived flavonoids, theobromine and magnesium [11]. It's been widely reported that the theobromine, an essential phytocompounds of *T. cacao*, scavenges reactive oxygen species (ROS) generated within the skin as a consequence of UV exposure, hormonal dysfunctioning, aging and other environmental factors which interfere with altered signaling pathways in the skin as a consequence [12] [13] [14]. Moreover, Cocoa's polyphenolic phytocompounds also help in delaying and reducing, the age-related brain impairments, including cognitive deficits in normal aging and perhaps neurodegenerative diseases too. It's also been reported to provide protection against nerve injuries, inflammation and persuade coronary vasodilatations and intensify endothelial nitric oxide content to prompt vascular slackening, expand vascular utility and also decline platelet linkage [15] [16] [17]. In the same way, polyphenols have anti-inflammatory action, over the inhibition of dissimilar transcription elements and cytokines [18] [19]. However, the current developments in pharmacological data has enhanced the competence of fragments to permit over the skin by refining the pharmacokinetics of medications; but then there is no sufficient and appropriate vehicle, that stayed de-

veloped, to safeguard intense, stable and uninterrupted sustained drug delivery using non-invasive and patient compliant techniques [20] [21] [22]. Most of the dermal formulations developed are either cream or lotion based which doesn't permeate well through the skin and are less absorptive, therefore, other pharmaceutical intervention which can enhance the absorption and permeation through the multi-layered dermal structure is always preferred [22].

Hydrogels are unique materials that are elastic in nature and have a broad range of applications in cosmetics, medicine, biomaterials and food technologies [23]. They allow higher degree of dermal hydration, drug transportation and dissolution, proving to be a suitable candidate for transdermal application. Many therapeutic agents such as curcumin, turmeric, neem and sandalwood have been loaded in hydrogels for the purpose of efficient skin damage healing and treating infections [24]. It was suggested that the combination of bioactivity of cocoa encapsulated in hydrogel system may prove beneficial in treating skin infections, reducing skin oxidative stress and has a potential for cutaneous healing ability. Therefore, in the present study, we opted for developing *T. cacao* extract loaded hydrogel formulation that will be more patient compliant, would have higher impregnation and absorbability for transdermal application [24] [25] [26].

2. Materials and Methodology

Materials

Theobroma cacao purified extract was provided by AESTHETIC company, Saharanpur, Uttar Pradesh, India as a gift sample and Folin-Ciocalteu reagent, gallic acid, Carbopol-940 and glycerin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), H_2O_2 reagent, Griess reagent, sodium nitroprusside were procured from Sigma Aldrich (USA). All other chemicals used in the experiments were of analytical grade.

3. Methodology

3.1. Sample Preparation (*T. cacao* Extract)

The *T. cacao* extract was prepared by defatting the cocoa butter from the extract completely with no loss of polyphenolic content as reported by P. Camasca, meeting the current regulation and ordinance standards regarding cocoa processing [27]. Then the cocoa extract (2 grams) was mixed with 10 ml of n-hexane solution and kept in a tightly capped reagent bottle on shaker for 24 hours. Thereafter, the defatted extract solution was subjected for ultra-sonication (25 kHz) for 30 minutes followed by a filtration and drying of filtrate in a vacuum-dryer (DZF-6050) at 37˚C and stored at −20˚C for further use [28] [29] [30].

3.2. Total Phenolic Estimation

Phenols react with phosphomolybdic acid in Folin-Ciocalteu chemical in an al-

kaline medium to impart a blue-colored complex (molybdenum blue) which is then estimated and calculated spectrophotometrically at 765 nm [31]. The amount of total phenolic in *T. cacao* extracts (TCE) was determined by adding 1 ml of Folin-Ciocalteu reagent in 2 ml TCE followed by the addition of 0.5 ml of Na_2CO_3 solution. The reaction mixture was then incubated for 30 minutes; thereafter the absorbance was recorded at 765 nm. The gallic acid was taken as a standard and the values after calculation were expressed as gallic acid equivalents (GAE) [32].

3.3. Total Flavonoid Estimation

For quantifying the total flavonoid content in the given TCE sample, they were subjected for the aluminium chloride ($AlCl_3$) colorimetric method as reported by Lamuela-Raventós, A. Tornero *et al*. In this assay, there is a development of acid constant multiplexes with C-4, C-3, and C-5 keto groups of flavones when mixed with $AlCl_3$ and subsequently, leads to formation of aluminium chloride rings which are acid stable complex with ortho-dihydroxyl groups [33]. Gallic acid was used as a standard and different concentrations TCE (10 - 50 µg/ml) was prepared in methanol and to this 0.2 ml aluminium chloride, 0.1 ml potassium acetate solution and 2.5 ml distilled water (DW) was added. After the 30 minutes of incubation, the absorbance of the samples was measured at 415 nm [34] [35].

3.4. Preparation of *T. cacao* Extract Loaded Hydrogel (TCHG)

The hydrogels are prepared and molded through various biochemical or physical crosslinks that construct the system and physical steadiness. These physical cross linkages include van der Waals interfaces or hydrogen attachment. Here in the present study, we have prepared the *T. cacao* defatted extract solution (TCE) loaded polymeric hydrogel by dispersion method [36] [37]. To prepare the gel carbopol-940 (10 µg/ml) was dispersed in water for two hours to get swollen up gelling mixture under constant mild stirring [38]. Further, the prescribed amount of TCE (10 µg/ml) was added in the gelling mixture along with triethanolamine (5 µg/ml) and glycerin (30 µg/ml) to maintain the pH and improve viscosity of the prepared hydrogel (TCHG). The optimization of the prepared formulation was done on the basis of various physicochemical as well as release kinetics parameters [38] [39].

4. Characterization of TCHG

4.1. Measurement of Physicochemical Parameters of TCHG (pH, Viscosity and Appearance)

The pH of all prepared TCHG formulations was measured by preparing 1% aqueous solution of the same and measuring it by using a calibrated digital pH meter (Mettler Toledo MP 220, Greifensee, Switzerland) at 37˚C. Subsequently, these formulations were also analyzed for viscosity which was determined by viscometer (Brookfield viscometer) at various angular velocities and then TCHG

formulations were inspected visually for the colour, odor and consistency [40] [41] [42].

4.2. Determination of Spreadability

The spreadability analysis of optimized hydrogel formulation (TCHG) was done by determining range of the expanded diameter of TCHG, when 500 mg of the same was placed between the two glass plates. The glass plates were left for 1 minute so that the gel can spread over maximally within a circle. Thereafter, the attained diameter of the spread gel on glass plates was measured. The shorter the time interval, the better will be the spreading coefficients [42].

4.3. Scanning Electron Microscopy Analysis (SEM)

The surface morphology and shape of the optimized TCHG was analyzed by SEM (Scanning electron microscopy). The sample for the same was prepared by mounting the hydrogel with adhesive tape which was conductive in nature on the aluminium surface followed by coating the surface with gold sputtering under vacuum condition and the surface morphology was observed on SEM (ZEISS, EVO 18, Germany) at 15 KV voltage [43] [44].

5. Antioxidant Analysis

5.1. DPPH (2,2-Diphenyl-1-Picrylhydrazyl) Assay

DPPH is widely used antioxidant assay for analyzing the scavenging ability of a compound against free radicals [45]. The antioxidative effect in this assay is measured on the basis of fading away of DPPH (free radical) in test samples which turns purple to yellow color as soon as the DPPH formed by absorption of hydrogen from the antioxidant reduces. Similarly, here in the experiment, 0.5 ml of DPPH solution was added in 500 µl of the test samples (AA, TCE and TCHG) prepared in different concentrations (10 - 50 µl) and incubated for 30 minutes at 37˚C followed by recording the λ maxima at 517 nm [46] [47] [48].

The scavenging ability (%) of each test samples were calculated by below mentioned formula:

$$\text{Scavenging activity}(\%) = \frac{A_{(\text{OC})} - A_{(\text{OS})}}{A_{(\text{OC})}} \times 100$$

where, $A_{(\text{OC})}$ and $A_{(\text{OS})}$ refer to the absorbance of the control and sample at 517 nm.

5.2. ABTS (2,2-Azinobis (3-Ethylbenzothiazoline-6-Sulfonic Acid)) Assay

The antioxidative ability of the test samples was also estimated by ABTS assay, where ABTS is generated by persulphate oxidation of 2,2-azinobis (3-ethylbenzoline-6-sulphonic acid) radical (ABTS2•), which has a characteristic long wavelength absorption spectrum, measured at 745 nm [49]. The activity was es-

timated when ABTS radical cations ($ABTS^{+}$) were produced by reacting ABTS with 10 ml of 2.45 mmol·L^{-1} of potassium persulphate and allowing the mixture to stand for 12 - 16 hours in dark at 37˚C before use. Then in 500 µl of the test samples (AA, TCE and TCHG) at different concentrations (10 - 50 µl), 4 ml of diluted ABTS solution was added and after 30 minutes, absorbance readings were taken at 745 nm [50].

The calculation for scavenging ability (%) of each test samples is mentioned below:

$$\text{Scavenging activity}(\%) = \frac{A_{(\text{control})} - A_{(\text{test sample})}}{A_{(\text{control})}} \times 100$$

where $A_{(\text{control})}$ and $A_{(\text{test sample})}$ refers to the absorbance of the control and sample at 745 nm.

5.3. Hydrogen Peroxide Scavenging Ability (H_2O_2 Assay)

Hydrogen peroxides (H_2O_2) are the free radical reactive oxygen species (ROS) which are produced as the by-products of normal aerobic metabolism and increases during a stressed condition, infections, exercise, radiation etc. H_2O_2 is not toxic itself but eventually, under the mentioned conditions may get converted into even more toxic radicals (hydroxyl radicals) and thus, its removal is important [51]. H_2O_2 scavenging activity is based on the direct UV-Vis absorption measurement at 230 nm (A_{230}). For assay, in the different concentration of test samples (AA, TCE and TCHG) ranging from 10 - 50 µg/ml, 500 µl of H_2O_2 was added in of the test samples and incubated for 10 minutes at room temperature [52] [53] [54] [55].

The absorbance was measured at 230 nm and calculated as:

$$\text{Scavenging activity}(\%) = \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \times 100$$

where absorbance of the control and sample is at 230 nm.

5.4. Nitric Oxide (NO) Scavenging Activity

NO assay was originally described by Griess in 1879 and involves the chemical reaction between sulfanilamide and naphthyl ethylenediamine dihydrochloride (NED) under acidic conditions [56]. Nitric oxide scavenging activity is based on the diazotization reactions which lead to the formation of chromophoricazo-derivative of sulphanilamide and naphthyl ethylenediamine dihydrochloride (NED). The NO scavenging activity was estimated by taking 0.25 ml of 10 mM sodium nitroprusside dissolved in phosphate buffer solution (pH 7.2). Different concentrations of the test samples (10 - 50 µl/ml) were incubated for 3 hours at 37˚C with 500 µl of Griess reagent [57] [58].

The absorbance was measured at 546 nm and calculated as following:

$$\text{Scavenging activity}(\%) = \frac{I_O - I_1}{I_O} \times 100$$

where I_O and I_1 refer to the absorbance of the blank and sample respectively at 546 nm.

5.5. Antimicrobial Activity by Agar Well-Diffusion Assay

The TCE and TCHG were screened for their antimicrobial potential using agar well diffusion assay. The microbial cultures *Micrococcus Luteus* (MTCC 106), *Pseudomonas fluorescens* (MTCC 2421), *Bacillus licheniformis* (MTCC 1251), *Rhizopus oryzae* (25923), *Aspergillus niger* (10535), *Trichoderma harzianum* (MTCC 5179) were analyzed for anti-microbial sensitivity of both the test samples (TCE and TCHG) in comparison to the antibiotics—streptomycin or amphotericin B (Positive control) [58] [59] [60]. On the soft agar plates, sterile well of 9 mm each was punched and infused with 200 µl of the test samples (Aqueous *Theobroma cacao* extract (TCE), *Theobroma cacao* Hydrogel (TCHG), gel without extract (HG) and distilled water (DW)). Streptomycin or amphotericin B was selected as a positive control for bacterial and fungal plates (200 µl) respectively [61]. The plates were incubated at 37˚C for 24 - 48 h. Thereafter, antimicrobial activity was determined by measuring the diameter of zones of inhibition in mm [62]. The means and standard deviation (±SD) of the diameter of zones of growth inhibitions for the treatments are shown in results [63].

6. Results

6.1. Phytocompounds Estimation

The presence of the total phenolic content in *T. cacao* extract was determined by the Folin-Ciocalteu method, the total phenolic content in the extract was found to be 97.58 ± 1.38 mg/ml. Similarly, the presence of total flavonoids content in TCE was estimated by $AlCl_3$ method and was found to be 68.02 ± 0.59 mg/ml, after plotting the standard graph with gallic acid.

6.2. Fabrication and Optimization of *T. Cacao* Hydrogel

The *T. cacao* extract loaded hydrogel (TCHG) was fabricated by using Carbopol-940 along with the hydro-dispersion of cocoa extract and glycerin as shown in Table 1 (Fabrication and optimization of hydrogel using Carbopol 940 at various concentrations). The drug and polymer ratio were optimized at different concentrations in variable batches (F1, F2, F3, and F4) followed by-physical parameters like pH, viscosity, appearance, homogeneity and dug release content analysis.

Table 1. Fabrication and optimization of hydrogel using Carbopol 940 at various concentrations.

Name of ingredient	F1	F2	F3	F4
Carbopol 940	0.50%	1%	1.50%	2%
Glycerin	30 ml	30 ml	30 ml	30 ml
Cocoa extract (TCE)	1%	2%	3%	4%

The hydrogel inspected for its pH had all values ranging between 6.4 ± 0.26 - 6.84 ± 0.49, considered safe as per the GRAS (Generally regarded as safe) limits to avoid skin irritation after application. The viscosity of hydrogel formulations ranged from 29.406 ± 1.08 cps to 51.814 ± 1.5 cps and Formulation batch (F4) showed the maximum viscosity as compared to the other formulations. On the contrary, F2 batch formulation exhibited appropriate and desired viscosity of the hydrogel formulated (TCHG) as discussed by Murthy *et al*., 2012 (Table 2) [41] [42]. Also, it was being observed that all the prepared and optimized formulations (F1 - F4) were homogenous in nature and there was no sign of phase separation, precipitation or agglomeration observed. Thereafter, the results obtained after spreadability testing presented that formulation F4 had minimum (11.94 ± 0.84 g·cm/s) and F2 formulation has maximum (27.49 ± 0.30 g·cm/s) spreading extension in accordance to their respective viscosities, as discussed earlier (refer to Table 2). The prepared and optimized all the hydrogel formulations were stored at room temperature for further use.

Table 2. Rheological parameters.

Formulation code	F1	F2	F3	F4
pH	6.4 ± 0.26	6.68 ± 0.54	6.8 ± 1.26	6.84 ± 0.49
Viscosity (cps)	29.406 ± 1.08	35.965 ± 0.56	42.510 ± 1.3	51.814 ± 1.5
Spreadability (g·cm/s)	23.10 ± 0.07	27.49 ± 0.30	15.78 ± 0.67	11.94 ± 0.84

6.3. *In-Vitro* Drug Release Kinetics

The *in-vitro* drug release kinetics of all the batches of TCHG were further assessed for cumulative amount of phenolic content released in 12 hours. It was found that the *in-vitro* drug release was best explained by Hixon's equation, as the plots showed the highest linearity (R^2 = 0.9931), followed by Higuchi's equation (R^2 = 0.9912). As it is clear from Table 3 below that the maximum release of drug was obtained at 12th hour in F2 formulation. The linear profile (approx.) as shown in Figure 1 was seen in all the formulations. The expected characteristics of hydro particles of sustained release were verified. The compound release from the hydrogel were clearly observed to attain 90.94% in 12 hours and on the order of release rate obtained for all the formulations (Table 4) we can rank them in the following descending order F2 > F3 > F4 > F1.

Table 3 illustrates the profile of *in-vitro* release of *T. cacao* extract from various formulations of TCHG hydrogel. It is clearly observed that over 90% of *T. Cacao* was released from F2 formulation within 12 h, whereas F1, F3 and F4 released less than 80% of drug during this period. These three formulations (F1, F3 and F4) had a consistent release rate over 8 h, after which the release rate gradually decreased and became constant with only a slight fluctuation. Moreover, F2 had the highest release rate, while F1 exhibited the lowest rate, indicating that the addition of polymer reduces the rate of release of the drug. This was primar-

ily because of the presence of carbopol-940 which raised the viscosity in the system and the high viscosity served to retard the diffusion of the drug. Additionally, the dialysis membrane method adopted in the present study allowed permeation of water molecules across the dialysis membrane. Considerable structural changes thus occurred, including alteration of shape and size distribution of the pores, due to progressive swelling of hydrophilic composite hydrogels, resulting in an increase in the tortuosity during the diffusional release of the drug [64].

Table 3. Drug release kinetics of hydrogel formulations.

Time (hours)	F1 (% drug release)	F2 (% drug release)	F3 (% drug release)	F4 (% drug release)
0	0.05	**0.13**	0.11	0.09
1	16.16	**22.97**	21.34	20.55
2	32.44	**38.92**	36.4	33.55
3	47.94	**52.23**	51.22	47.49
4	53.61	**60.32**	58.15	55.61
5	59.63	**65.49**	64.6	63.79
6	64.59	**75.62**	73.55	72.29
7	73.41	**81.88**	79.35	78.17
8	78.11	**88.29**	80.36	79.20
9	78.26	**89.03**	80.44	78.79
10	78.35	**89.96**	79.63	78.57
11	78.44	**90.57**	78.95	78.49
12	77.26	**90.94**	78.93	78.21

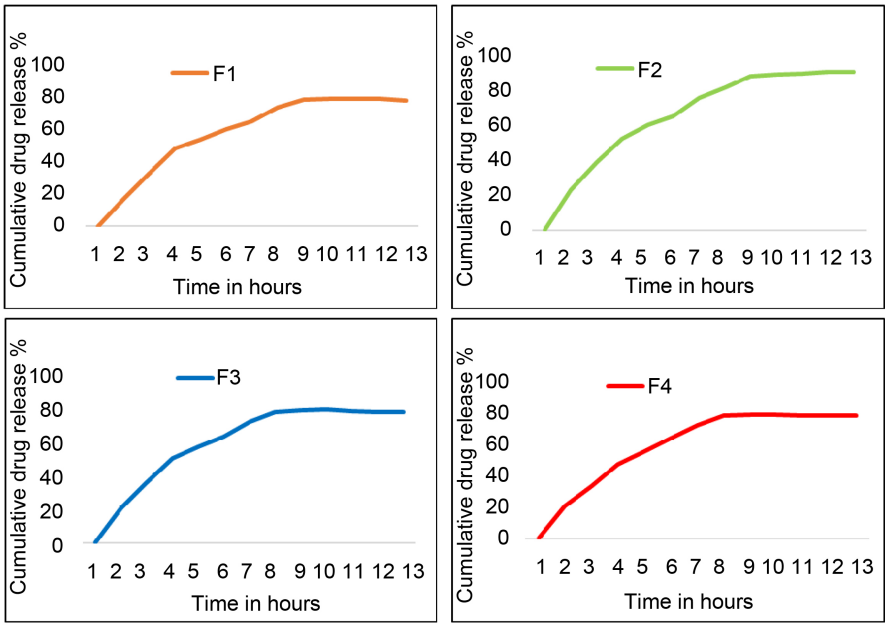

Figure 1. Drug release of F1, F2, F3, F4 formulations.

Table 4. Drug release model.

Formulation	Zero order	First order	Higuchi	Hixson-equation	Korsmeyer-Peppas
F2	0.985	0.913	0.909	0.985	0.781

6.4. Scanning Electron Microscopy (SEM)

The SEM analysis of morphology of TCHG (F2) depicted the uneven and apparently flaky surface appearance with average porosity, giving avenue for decent extract loading capacity followed by controlled release characteristics. The balanced viscosity in the TCHG (F2) formulation might have led to an efficient inter micellar continuities in the hydrogel composites resulting in increased storage coefficients and long hydrophilic chain as shown in **Figure 2**.

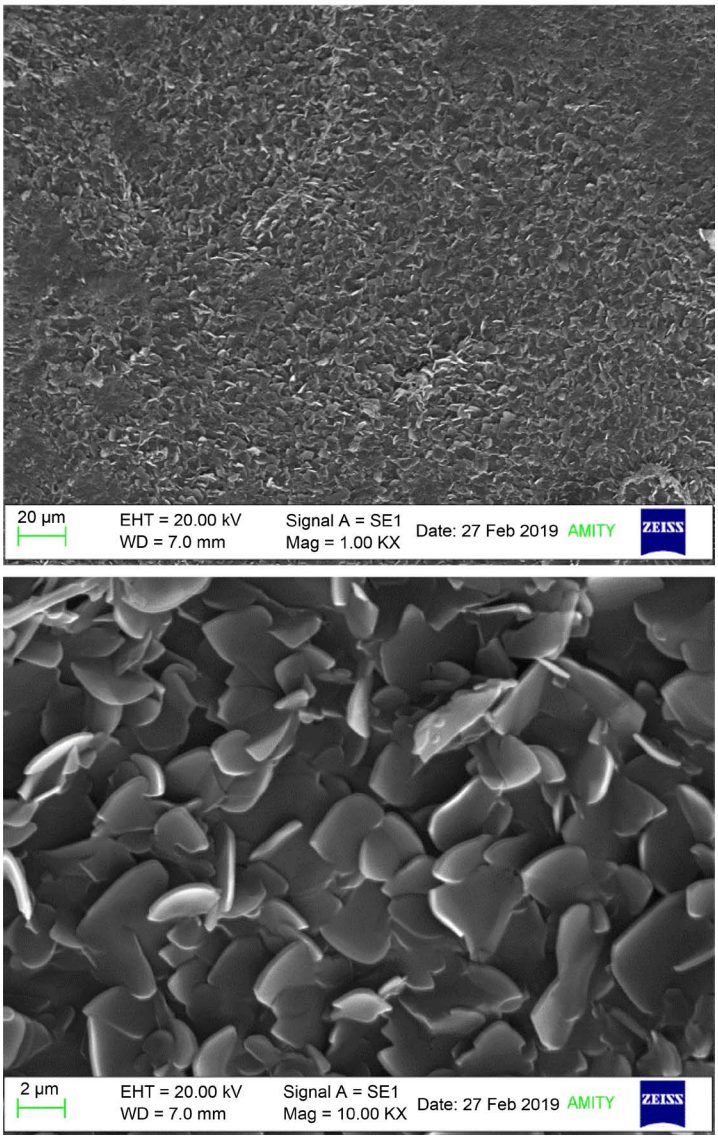

Figure 2. SEM analysis of TCHG at the magnification of 1.00 KX and 10.00 KX respectively.

6.5. Antioxidant (AO) and Antiradical Analysis

The standard graph of Ascorbic acid (AA) at different concentration (10 - 50 µg/ml), and was analyzed 517 nm. After plotting the values on the standard graph the R^2 value was found out to be 0.996. Thereafter the same the antioxidant activity was evaluated for cocoa gel as well as the extract.

6.6. Radical Scavenging Assay (DPPH)

The antioxidant activity was plotted against various concentration (10 - 50 µg/ml) of test samples *Theobroma cacao* extract (TCE) and cocoa gel (TCHG). The antioxidant activity of gel was found out to be maximum at the concentration of 50 µg/ml which was 92.15% ± 1.42% whereas; the extract shows the % antioxidant activity of 40.31% ± 1.13% at the same concentration as shown in Figure 3. Therefore, it was well reflected from the results that cocoa gel (TCHG) has a maximum antioxidant activity followed by the standard (AA) as shown in Table 5.

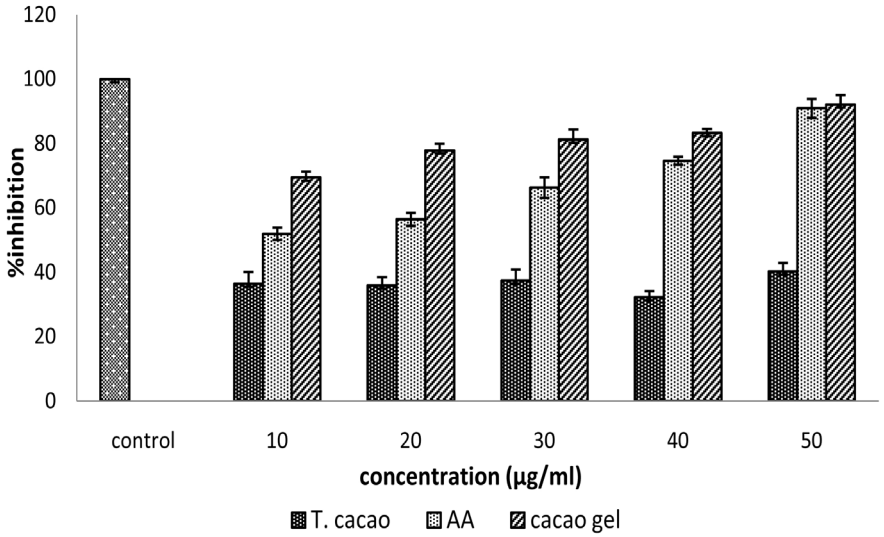

Figure 3. The Graph depicting DPPH Radical scavenging activity (%) of *T. cacao* (TCE), cacao gel (TCHG) and standard (AA) at different concentration.

Table 5. Showing the percentage of DPPH radical scavenging activity.

Test sample	Radical scavenging activity
Theobroma cacao	48.72% ± 1.48%
Ascorbic acid	76.85%
Cocoa gel	80.93% ± 1.81%

6.7. 2,2-Azinobis (3-Ethylbenzothiazoline-6-Sulfonic Acid) Radical Assay (ABTS)

The antioxidant activity was plotted against various concentration (10 - 50

μg/ml) of test samples *Theobroma cacao* extract (TCE) as well as cocoa gel (TCHG). The antioxidant activity of gel was found out to be maximum at the concentration of 50 μg/ml which was 95.27% ± 1.69% whereas; the extract shows the % antioxidant activity of 60.12% ± 1.23% at the same concentration as shown in Figure 4. Therefore, it was well reflected from the results that cocoa gel (TCHG) has a maximum antioxidant activity followed by extract and the standard AA as shown in Table 6.

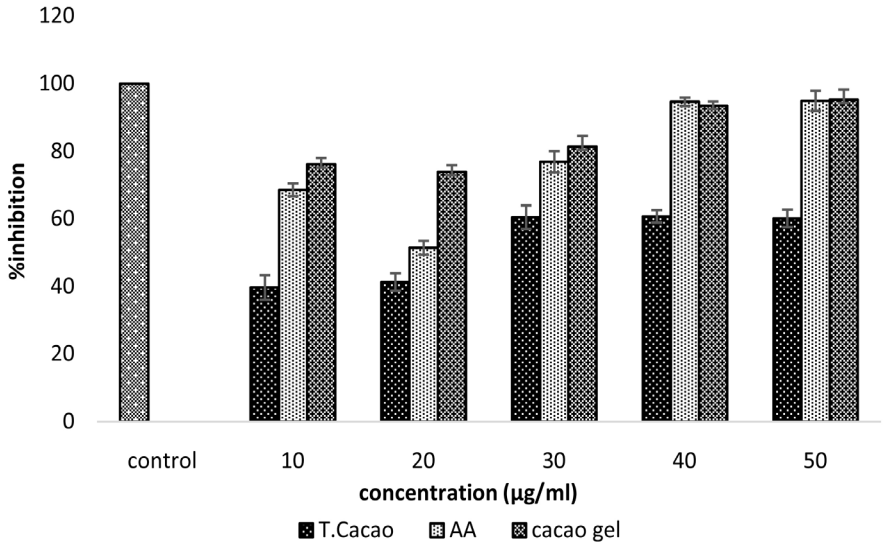

Figure 4. The Graph depicting ABTS scavenging activity (%) of *T. cacao* extract (TCE), cacao gel (TCHG) and standard (AA) at different conc.

Table 6. Showing the percentage of ABTS radical scavenging activity.

Test sample	Radical scavenging activity—ABTS
Theobroma cacao	56.25% ± 1.51%
Cocoa gel	79.39% ± 1.63%
Ascorbic acid	74%

6.8. Hydrogen Peroxide Scavenging Activity—H_2O_2 Assay

The scavenging behavior of the test samples *T. cacao* extract (TCE) as well as cocoa gel (TCHG) was dose-dependent as it increases with the increase in concentration. The antioxidant activity was plotted against various concentration (10 - 50 μg/ml) of test samples *Theobroma cacao* extract (TCE) as well as cocoa gel (TCHG). The antioxidant activity of gel was found out to be maximum at the concentration of 30 μg/ml which was 98.12% ± 0.42% whereas; the extract shows the % antioxidant activity of 57.13% ± 1.13% at the same concentration as shown in Figure 5. Therefore, it was well reflected from the results that cocoa gel (TCHG) has a maximum antioxidant activity followed by extract (TCE) and the standard (AA) as shown in Table 7.

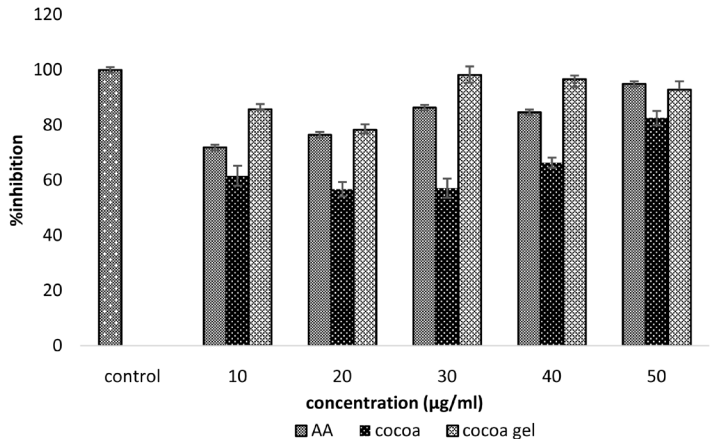

Figure 5. The Graph Depicting H_2O_2 scavenging activity (%) of cocoa extract (TCE), cocoa gel (TCHG) and standard (AA) at different concentration.

Table 7. Showing percentage of H_2O_2 scavenging activity.

Test sample	scavenging activity—H_2O_2 assay
Theobroma cacao	72.90% ± 1.51%
Cacao gel	91.23% ± 0.55%
Ascorbic acid	78.85%

6.9. Nitric Oxide Scavenging Activity—NO Assay

The antioxidant activity was plotted against various concentration (10 - 50 µg/ml) of test samples *Theobroma cacao* extract (TCE) as well as cocoa gel (TCHG). The antioxidant activity of gel was found out to be maximum at the concentration of 50 µg/ml which was 92.54% ± 0.69% whereas; the extract shows the % antioxidant activity of 61.5% ± 1.43% at the same concentration as shown in **Figure 6**. Therefore, it was well reflected from the results that cocoa gel (TCHG) has a maximum antioxidant activity followed by extract (TCE) and the standard (AA) as shown in **Table 8**.

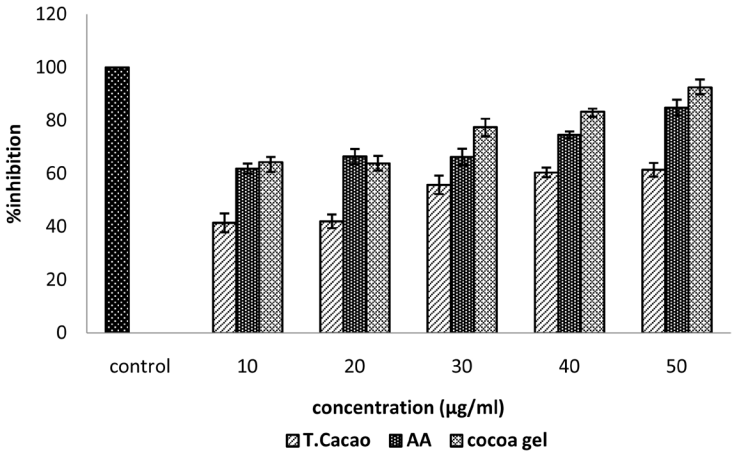

Figure 6. The Graph depicting NO scavenging activity (%) of *T. cacao* extract (TCE), cocoa gel (TCHG) and standard (AA) at different conc.

Table 8. Showing percentage of NO scavenging activity.

Test sample	scavenging activity—NO assay
Theobroma cacao	58.90% ± 1.51%
Cocoa gel	95.17% ± 1.14%
Ascorbic acid	85.16%

6.10. Antimicrobial Activity

When the samples were loaded in the agar plates, there occurs the diffusion of the sample into the well which leads to the inhibition of growth of microbes in and around the wells loaded with samples. These inhibitions are clearly seen in the plates after incubation of 24 hours to 72 hours for anti-bacterial and anti-fungal activity. These zones are marked and then measured in mm which determines the inhibition of the microbes by the test samples as shown in **Figure 7**. Out of the 3 bacterial strains used, maximum inhibition zones were found in the species of *Bacillus licheniformis*. These zones were measured as shown in **Table 9** and were further analyzed for MIC calculation as shown in **Table 10**. It was seen that the cocoa extract showed maximum activity against *Pseudomonas fluorescens*, *Bacillus licheniformis*, *A. niger*, *Trichoderma* as the maximum zones of inhibition were found in it but shows activity against rest of the strains too. Out of both bacterial and fungal strains cocoa showed distinct zones and was further reported for estimation of Minimum Inhibitory Concentration (MIC). MIC is calculated by the diameter of the zones of inhibition. The diameter of the inhibition zones is directly proportional to the concentration of the antibiotics.

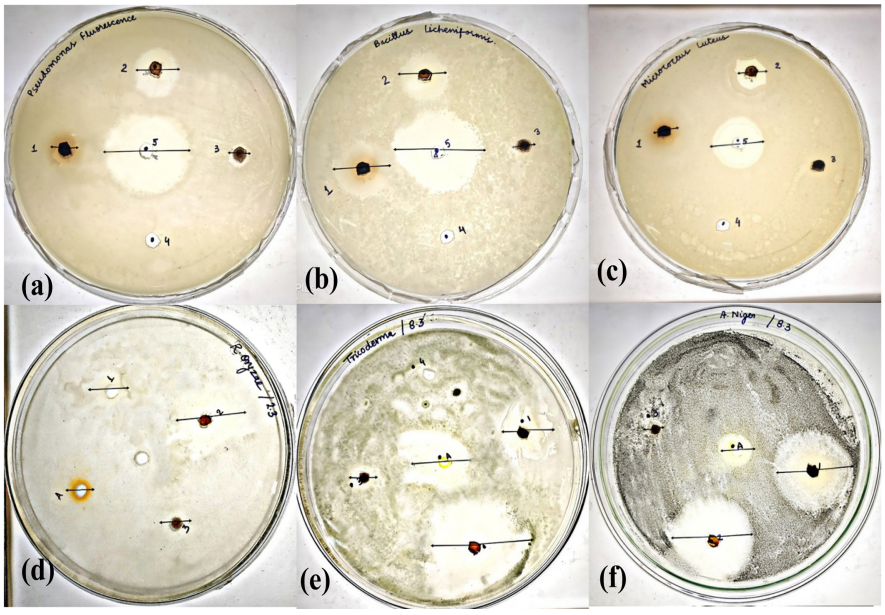

Figure 7. Zones of inhibition in microbial strains. (a) *Pseudomonas fluorescens*; (b) *Bacillus licheniformis*; (c) *Micrococcus luteus*; (d) *Rhizopus oryzae*; (e) *Trichoderma*; (f) *Aspergillus niger*. Where each well represents following test samples—1.TCE; 2. TCHG; 3. gel without extract HG; 4. distilled water DW; 5. Antibiotic.

Table 9. Zones of inhibition of different cocoa samples TCE, TCHG and HG on different microbial strains.

strains / Test sample	*Pseudomonas fluorescens*	*Bacillus licheniformis*	*Micrococcus luteus*	*Rhizopus oryzae*	*Trichoderma harzianum*	*Aspergillus niger*
TCE	25 ± 1.06 mm	25 ± 1.54 mm	15 ± 1.16 mm	14 ± 1.33 mm	45 ± 1.21 mm	45 ± 1.14 mm
TCHG	25 ± 1.39 mm	30 ± 1.16 mm	23 ± 0.96 mm	25 ± 1.73 mm	60 ± 0.09 mm	45 ± 1.7 mm
Gel without extract HG	12 ± 1.32 mm	3 ± 1.81 mm	2 ± 1.43 mm	8 ± 1.29 mm	12 ± 1.75 mm	0 ± 1.31 mm
Positive control (antibiotic)	40 ± 1.6 mm	50 ± 1.4 mm	35 ± 1.6 mm	10 ± 1.55 mm	40 ± 1.59 mm	20 ± 1.6 mm

Minimum Inhibitory Concentration:

$$X = (a - b)/2;$$

where a = diameter of the inhibition zones, b = diameter of the well.

Then plotting the relation between X^2 and log (concentration) and from the curve the MIC is calculated. The antilog of the value of the intercept falling on the X-axis determines the MIC of the sample [65].

Table 10. Minimum inhibitory concentration of test samples on different microbial strains.

organism	d	10^{χ}	MIC (µg/ml)
Pseudomonas fluorescens	1.39	$10^{1.397}$	24.94 ± 1.02
Bacillus licheniformis	1.4	$10^{1.4}$	25.11 ± 1.47
Micrococcus luteus	1.43	$10^{1.43}$	26.91 ± 1.89
Rhizopus oryzae	1.48	$10^{1.48}$	30.19 ± 0.39
Trichoderma	1.36	$10^{1.36}$	22.90 ± 0.69
Aspergillus niger	1.33	$10^{1.33}$	21.37 ± 0.58

7. Discussion

In the quantitative analysis of the cocoa extract which is done to confirm the presence of certain phyto-constituents as well quantify the amount of these phyto-chemicals in the plant. The quantification of metabolites helps determine the impact of presence and amount of heavy metal present in the sample on the over-all well-being of the plants [66]. Moreover, since these metabolites are responsible for imparting health benefits to the host upon consumption, thus it is essential to understand the extent of impact of these metals on plants to correlate their effect on humans [67].

This correlation is done by assessment and quantification of phytoconstituents in the plants which are the major toxicants being discussed. The rest of the analysis includes the estimation of total phenolics and flavonoid content [68]. It was observed and estimated by R. Gunawan *et al*., that there was high content of phenolics and flavonoids present in the sample of cocoa which shows many the-

rapeutic effects and has much more value in the same way the results obtained in this study was approximately near to the values as described above [69] [70]. Hence, the study of *Theobroma cacao* is validated to the best of knowledge.

Hydrogel formulation containing cocoa hydrogel was successfully prepared and showed effective as well as better carrier for the transdermal drug delivery. The formulated hydrogel was optimized for pH, viscosity, drug release, extrudability, homogeneity [71]. As per Ashwini Jaybhave *et al*., administration of this through dermal route bypasses the disadvantages of oral route and maintain the consistent plasma levels for the therapy of single dose [72]. Therefore, as proved by Pritam Nikam *et al*., the results showed that the gel had the ability to retain the original properties for the duration of 6 months which proved the hydrogel formulation to be better and cost-effective in comparison to oral doses [73] [74]. In the same way, the formulations reported in this study reflected the same effectiveness when compared to the work done during this thesis, hence the optimization of the hydrogel and the drug release kinetics were found nearly similar which proves the authenticity of the work [75] [76] [77].

There are various elements involved in the "antioxidant paradox," which has recently been analyzed by Halliwell *et al*. For example, the use of high doses of antioxidants may actually be detrimental, with lower doses being more beneficial, as is the case with catechin and epicatechin (Halliwell, 2009) [78] [79]. Moreover, the potential value of antioxidants is often low due to their low levels in cells and tissue, thus limiting their possible use as therapeutic drugs [80]. For example, in the therapeutic strategies to prevent progressive neuronal loss based on antioxidant activity, the antioxidant must be able to cross the blood brain barrier and be present at the respective brain region for neuroprotection or the transdermal layer for skin cancers (Aruoma, 2003) [81]. That being said, food, nutraceuticals, and complementary medicines facilitate the administration or ingestion of high quantities of antioxidant compounds, which can produce high or moderate levels of antioxidant agents in the blood and tissue [82] [83]. This protects the natural antioxidants from destruction and consequently reduces cell and tissue damage. To date, numerous studies on the antioxidant activity of plant extracts have been published [84]. Of these, Lee, Kim, Lee, and Lee *et al*. (2017) compared the antioxidant effects of cocoa extract against cocoa gel extract [85] [86]. These authors observed high activity for the cocoa extract and gel in both ABTS and DPPH tests as well as in NO, H_2O_2, most likely due to a high content in phenolics in it. In the same way, the results obtained during this thesis work validated the study done [87] [88].

The use of *T. cacao* as an anti-microbial agent in many traditional medicinal system has indicated that *T. cacao* may be useful for specific infectious diseases [89]. Hence, there is no need to scientifically evaluate the plant so that it can be used as a novel anti-microbial agent [90]. There are various publications which report the anti-microbial property of *T. cacao* comparison of the present findings with previous publications is difficult. Plants are known to vary in their composition with changes in their habitat and climatic conditions [91]. The

methods used to check anti-microbial activity of various plant extracts and selection of the test organisms vary between publications. Moreover, a comprehensive report on the activity of *T. cacao* on skin infections causing pathogens is not available [92].

Therefore, the present study indicates that *T. cacao* extracts and gel both have a potent anti-microbial activity against various skin pathogens [93] [94] [95]. However, MIC values (**Table 10**) indicates that it has higher action against fungal strains than that of the bacterial strains [96]. Moreover, gel extract has a more effective action towards the microbial strains as compared to pure extract which was evident from the MIC values [97]. This indicates that the polyphenolic components of *T. cacao* extract, which are responsible for its anti-microbial activity, are more effective as gel than that of the powder [98]. However, the study reported by Chou *et al.*, *P. fluorescens* has been found to be more sensitive toward the cocoa extract which confirms the result obtained for the gel extract but the same was not found for the powder extract [99].

Cocoa hydrogel is being prepared using 1% carbopol-940 as a gelling agent, as it shows better drug release as compared to other formulations, *i.e.* 90.94% and the formulation follows Hixon-Crowell model of drug release kinetics. Appearance of the gel was found to be homogenous with no grittiness and brown in colour. The pH was found to be 6.68 ± 0.54 and viscosity 35.965 ± 0.56.

8. Conclusions

T. cacao has been proved to be very effective in managing many disorders and infections which are associated with skin. Phytochemical studies of *T. cacao* have shown the presence of various versatile and important phytoconstituents such as flavonoids, phenols, alkaloids and saponins. Out of which maximum quantity was estimated for phenolics which was 97.58% ± 1.51%.

The hydrogel prepared showed a good release of drug and had increased bioavailability. The *ex-vivo* studies of cocoa hydrogel performed showed the maximum drug release of cocoa extract after 12 consecutive hours which proves the effectiveness of the hydrogel. Therefore, the hydrogel containing cocoa proved for *in-vitro* anti-microbial activity and anti-cancer activity.

On evaluating the antioxidant and antiradical properties of cocoa hydrogel by DPPH, ABTS, NO, H_2O_2, it showed that cocoa hydrogel has better radical scavenging effect than the standard of ascorbic acid (AA).

Further results affirmed our study for the activity of cocoa gel against some sensitive bacterial and fungal strains. The anti-bacterial study of cocoa was established against *M. leuteus* (MIC = 26.91 ± 1.89 µg/ml), *B. licheniformis* (MIC = 25.11 ± 1.47 µg/ml), *P. fluorescens* (MIC = 24.94 ± 1.02 µg/ml) and for fungal strains *R. oryzae* (MIC = 30.19 ± 0.39 µg/ml), *Trichoderma* (22.90 ± 0.69 µg/ml), *A. niger* (21.37 ± 0.58 µg/ml). It further reflects that there can be a hope for the development of many novel products or templates from such plants which may serve for the production of synthetically improved medicinal and therapeutic agents for various disorders.

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Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization, SHRIYA AGARWAL, VANDANA TYAGI and MANISHA SINGH Methodology, SHRIYA AGARWAL and MANISHA SINGH; Software, SHRIYA AGARWAL, VANDANA TYAGI, MUGDHA AGARWAL; Validation, SHRIYA AGARWAL; Formal Analysis, MANISHA SINGH, RACHANA; Investigation, MANISHA SINGH; Resources, SHRIYA AGARWAL, VANDANA TYAGI; Data Curation, SHRIYA AGARWAL, MANISHA SINGH, MUGDHA AGARWAL; Writing-Original Draft Preparation, SHRIYA AGARWAL, MANISHA SINGH; Writing-Review and Editing, SHRIYA AGARWAL and MANISHA SINGH; Visualization, MANISHA SINGH; Supervision, MANISHA SINGH; Project Administration, MANISHA SINGH.

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