Biotransformation in Combination of Traditional Medicinal Plants: The Effect of Wickerhamomyces Anomalous on the Antidiabetic Potential and Metabolite Profiling

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Abstract

**Background:** Biochemical transformation is a green and efficient strategy that can be of help in case of potentiation of herbal treatments. Diabetes requires daily therapeutic interventions and the use of herbals can be safe and effective alternatives however higher doses of herbs can cause much inconvenience. The potentiation of traditional medicinal plants based polyherbal combinations to reduce the dosage can be an attractive approach.

**Methods:** The present study explored the biochemical transformation of a polyherbal extract derived from *Syzygium cumini* seed, *Mangifera indica* seed kernel, *Momordica charantia* fruit and *Gymnema sylvestre* leaf using *W. anomalus*. The polyherbal extract was chromatographed and evaluated for antidiabetic potential, Pre and Post fermentation. Also, the fermentation kinetics was assessed as *W. anomalus* can produce ethanol which can be self-limiting. **Results:** The extract produced no behavioral or physical changes in animals, confirming the safety in acute toxic exposure. The chromatographic analysis showed good presence of Hesperidin, Mangiferin and Corilagin in non-fermented extract which was significantly reduced and content of Apigenin, Luteolin, Quercetin, kaempferol and Isorhamnetin were increased after fermentation.

**Conclusions:** The polyherbal extract showed good antidiabetic activity however fermented polyherbal extract showed a similar effect at half the dosage. The effects are also corroborated by histological studies.

1. Introduction

Fermented traditional medicine represent an important part of the worldwide. Whole-cell biotransformation has been reported as an important tool that can be used to modify the chemical composition of the substrate medium. The chemical analysis of the substrate and product gives an idea about the enzyme structure as well as the metabolic infrastructure of the microorganism. Also, the use of microbes can yield a specific set of a substrate with the use of milder conditions which further enables simple downstream processing to afford a usable concoction. The successful use of a combinatorial fraction also validates the use of a systems biology approach in herbal-based preparation as it provides a holistic response [1–5].

Biotransformation using pure microbial cells is the perfect choice because of specific reasons like good surface-volume ratio, high growth rate, the higher rate of metabolism leading to an efficient transformation of substrates under sterile conditions [3]. Microbial metabolism comprises Primary metabolism, which is in control of cell capacity, and secondary metabolism, which utilizing existing metabolic pathways produces substances for the acclimatization of the organism to its environment. Secondary metabolism generates small molecular weight metabolites that are not necessary for growth but that offer various advantages and may occasionally have medical/industrial significance [4, 5].
Diabetes is speedily emerging as the most common ailment in the world. Being a chronic disorder, Diabetes needs lifelong interventions. Currently available oral hypoglycemic agents need extreme caution and regularity to maintain the sugar level. The use of herbal therapy can help in maintaining the sugar level however the dose constraints affect its use in the long term. Antidiabetic activity of polyherbal formulations has been evaluated to discover more effective and safe therapeutic agents. Flavonoids being an omnipresent category have the widest circulation in the plant kingdom. Further various flavonoids have been established to possess the antidiabetic activity along with antioxidant, free radical scavenging, anti-inflammatory, anti-carcinogenic and antimutagenic properties combined with their ability to modify cellular metabolism. They are known inhibitors of COX (Cyclooxygenase), LOX (Lipoxygenase), XO (Xanthine oxidase) and phosphoinositide 3-kinase \[1\].

Gino et al evaluated the combinatorial effect of a poly-herbal combination having a hypoglycemic and hypolipidemic agent. The antidiabetic activity of combination of Salacia oblonga, Tinospora cordifolia, Emblica officinalis, Curcuma longa and Gymnema sylvestre was determined in streptozotocin (55 mg/kg, i.p.)-induced diabetic rats, at a dose of 100 mg/kg \[6\].

Nagja et al determined the anti-diabetic activity of a polyherbal formulation (containing Eugenia jambolana, Gymnema sylvestre, Momordica charantia and Andrographis paniculata and sodhana processed extract of Myristica fragrans) in streptozotocin-induced type 2 diabetic rats \[7\]. Mazlan et al studied biotransformation of M. charantia fruit juice via lactic acid bacterium fermentation and observed reduced bitterness, reduced sugar content and production of aglycones and other metabolites as well as improvement in its α-glucosidase inhibition compared with the fresh, non-fermented juice \[8\]. Petchi et al. evaluated the synergistic effect of Glycosmis pentaphylla, Tridax procumbens, and Mangifera indica in diabetes \[9\]. Vidya et al (2013) demonstrated the hypoglycemic and antidiabetic activity of a polyherbal formulation, in both normal and experimentally induced hyperglycemic (Streptozotocin) rats \[2\].

Fermentative biotransformation is an ecofriendly and efficient strategy of chemical modifications that have the potential to alter the therapeutic outcomes of the substrates. Wickerhamomyces anomalus is a biocontrol yeast and contains several enzymes e.g. invertase, phytase, glycosidase etc. Diabetes needing lifelong interventions must have effective, safe and sustainable therapeutic options i.e. polyherbal extract fermented with W. anomalus.

The present study explores the phytochemical and therapeutic outcome of fermentative biotransformation in polyherbal extract of a composite extract derived from Syzygium cumini seed; Momordica charantia fruits, Mangifera indica seed kernel, Gymnema sylvestre leaves. Wickerhamomyces anomalus (Strain no. MTCC-4133) was utilized for fermentative biotransformation. The fermentation process was further documented for kinetics and the flavonoid profile of fermented extract was quantitatively compared with non-fermented extract using HPTLC profiling. Fermented extract (FE) and non-fermented extract (NFE) were assessed for the acute toxicity in rats and antidiabetic potential in Streptozotocin-induced diabetes in rats, with Glibenclamide as reference standard. Also, the
histopathological changes in the vital organs i.e. kidney, pancreas and liver are studied to understand the cellular changes during treatment.

2. Materials & Methods

Plant materials (*Syzygium cumini* seed; *Momordica charantia* fruits, *Mangifera indica* seed kernel, *Gymnema sylvestre* leaves) were collected and authenticated by Dr. Anamika Tripathi, Associate Professor, Project Co-ordinator, NAMP, Department Of Botany, Hindu College, Moradabad, affiliated to Mahatma Jyotiba Phule, Rohilkhand University, Bareilly with Ref. No.- HC/Bot/PERL/209–2018.

2.1 Preparation of polyherbal Extract:

All the dried crude materials were physically cleaned, washed with water and then dried in an oven at 40°C for 3 h. It was then coarsely ground and sieved over 10# sieve. The retained material was utilized for extraction purposes [3].

All 4 ingredients were mixed in equal ratio (500 g each) in a polybag and then packed in a soxhlet apparatus. It was extracted for 72 h using water. The extract so obtained was concentrated utilizing a vacuum evaporator and finally dried to yield brown powder (396 g).

2.2 Fermentation of polyherbal Extract:

*Wickerhamomyzes anomalus* (Strain no. MTCC-4133) was utilized for fermentation. The lyophilized culture ampoule was revived on MYPG medium at 28 ± 0.5 °C for 72 h. The seed culture media was prepared and inoculated with slant media containing the fungal biomass and incubated at 28 ± 0.5 °C for 48 h. The dried polyherbal extract was dissolved in water to obtain 50% dispersion and employed as a substrate in the fermentation. The autoclaved 50% aqueous dispersion was inoculated with 5% v/v seed culture and placed in an incubator at 28 ± 0.5 °C. The duration for fermentation was selected as 72 h [5, 8].

1. **2.3 Extraction from Fermented polyherbal Extract:** After the completion of 72 h, the biomass was added with a 10 times volume of 1:1 mixture of water and methanol and filtered through 200# nylon cloth. The resultant extract was made methanol-free [4].

2. **2.4 Phytochemical screening**

The non-fermented and fermented extracts were subjected to initial phytochemical screening for determining their chemical composition. The absence or presence of various phytoconstituents like carbohydrates, alkaloids, steroids, saponins, tannins, flavonoids and terpenoids were identified by recommended test methods [10, 11].

2.5 Physicochemical characterization (Fermentation Kinetic Profile)
The fermentation kinetics was examined through the determination of pH, specific gravity, optical density, phenolic content, flavonoid content, reducing sugar and alcohol production at every 6 h up to 72 h. pH was taken as such whereas for reducing sugar and specific gravity, a filtered sample was used. For the determination of Optical density, the filtered medium was analyzed against 50% aq. dispersion as a blank. TPC was determined in relations of gallic acid equivalents. The flavonoid content was estimated in terms of Quercetin content. The ethanol content was assessed utilizing Gas Chromatography (GC) [12, 13].

The total phenolics were estimated utilizing the Folin and Ciocalteu method. A calibration curve was prepared using graded concentration (5-500 µg/ml) of Gallic acid. Test and standard readings were made utilizing a UV spectrophotometer at 765 nm together with the blank reagent. The phenolic content was expressed as gallic acid equivalents(GAE/g of dry plant material) based on a standard curve of gallic acid (5–500 µg/ml)[12].

Total flavonoid content was estimated by utilizing the aluminum chloride colorimetric technique. A standard calibration curve of quercetin was prepared using its graded concentration. The absorbance was estimated against blank at 420 nm wavelength with UV-spectrophotometer. The total flavonoid content was expressed as mg quercetin equivalent (QE)/g of dried plant material.

### 2.6 HPTLC Fingerprinting Analysis

#### 2.6.1 Sample Preparation: Standard stock solutions (1 mg mL⁻¹) of apigenin, luteolin, quercetin, kaempferol, isorhamnetin, hesperidin, mangiferin and corilagin were prepared in methanol. Working solutions (1–10 µg mL⁻¹) were prepared by appropriate dilutions of the stock solutions with methanol [14, 15].

Aqueous decoction and its fermented product were partitioned successively with hexane, chloroform, and ethyl acetate. The extracts were dried, and 50 mg ethyl acetate extract was dissolved in methanol to make the sample solution (10 mg/ml)

1. **2.6.2 Developing Solvent System**: The plates were developed in toluene–ethyl acetate–Acetic acid (5:4:1) for the first 5 compounds and Ethyl acetate-Formic acid-Acetic acid-Water (100:11;11:26) for last 3 compounds.

2. **2.6.3 Sample Application**: CAMAG HPTLC system (Muttenz, Switzerland) with a Linomat 5 sample applicator was employed for the analysis. HPTLC was done on precoated 60F254 silica gel HPTLC aluminum plates (E. Merck, Darmstadt, Germany). The respective sample (5 µL) was spotted as a 5 mm band by using the autosampler (fixed with a 100-µL Hamilton syringe).

3. **2.6.4 Chromatogram Development & Detection of Spots**: The developed plates were scanned at 254 and 366 nm using a spectro densitometer (with CAMAG Scanner 3) fitted with WINCATS planar chromatography manager (Version 1.3.0, CAMAG) software[16].

### 2.7 Acute toxicity study
Acute toxicity of the fermented extract (FE) and non-fermented extract (NFE) was determined to evaluate its toxicity potential. Rats were divided into a control and four test groups (n = 6). The test groups received FE and NFE orally at the doses of 1000, and 2000 mg/kg body weight respectively. After administration, the animals were kept in separate cages and were allowed to food and water ad libitum. The animals were then observed for possible behavioral changes, allergic reaction (skin rash, itching) and mortality for the next 72 h [2].

2.7.1 Antidiabetic activity

The protocol of the study was reviewed and approved by the Institutional Animal Ethical Committee (Registration Number: 1044/PO/Re/S/07/CPCSEA and Project Proposal no-ITS/10/IAEC/2018). All the animal activities were performed on male albino rats weighing 100–150 g. The rats were maintained on a normal fat diet and were fasted overnight with free access to water before any observation. During the experiment, the animals were divided into five groups of six animals in each group. Treatment was administered with a glass syringe and micro-suction canula no. 18.

2.7.2 Oral Glucose tolerance test

The blood glucose concentration of animals was measured at the beginning of the study. Then the rats were orally treated with 3 g/kg body weight glucose solution. The measurements were repeated after 30, 60, 120 and 180 minutes after the glucose load [2].

2.7.3 Induction of diabetes:

Animals have fasted for 24 hrs then a single intraperitoneal injection of freshly prepared Streptozotocin (65 mg/kg dissolved in 0.1 M citrate buffer, pH 4.5) was injected. The animals were left aside for 30 minutes and then a 10% glucose solution was placed in the cages for 48 hrs. The diabetes was confirmed by estimation of blood glucose level on the third day. Rats having Blood Glucose Level > 250 mg/dl were used for the study. For the antidiabetic activity, the animals were divided into five groups of six animals in each group. Treatment with FE and NFE started after the last Streptozotocin injection. Blood samples were collected at 0 day, 7 days, 14 days, 21 days and 28 days till the end of the study.

2.8 Histopathological Studies:

Rats were euthanized according to the CPCSEA guidelines. Pancreas, liver and kidney tissue were macroscopically examined and slices of each organ were fixed in 10% neutral buffered formalin with PBS. After the processes of dehydration, cleaning and infiltration, the organs were embedded in paraffin wax and section through a Micro-tome. The tissues were stained with hematoxylin and eosin. The slides were examined and captured with a digital microscope camera.

2.9 Statistical analysis

The data were expressed as mean ± SEM. The data of hypoglycemic activity, oral glucose tolerance test (OGTT), and antidiabetic activity were analyzed by one-way analysis of variance (ANOVA) followed by
Dunnett’s t-test for multiple comparisons. Values with $P < 0.05$ were considered significant.

3. Results & Discussion

Diabetic burden is taking a heavy toll on human health as well as the World economy. Since it’s not curable, its management using oral hypoglycemic is very much essential for enhancing the patient life’s quality. The use of herbal options can lower the dose of oral hypoglycemic providing better efficacy however the herbal therapeutics have a higher dosage so their potentiation will greatly help in reducing the dosage and enhancing the patient compliance.

Biotransformation is a process by which organic compounds are transformed from one form to another to reduce the persistence and toxicity of the chemical compounds. These transformations can be grouped under the categories of hydrolysis, reduction, oxidation, condensation, isomerization, introduction of functional groups and development of new carbon bonds.

3.1 Phytochemical Screening: Both the extracts (NFE and FE) were phytochemically screened for the presence of alkaloids, glycosides, steroids, terpenoids, phenolics etc. The observations are given in Table 1. Steroids were absent in both the extracts whereas alkaloids, carbohydrates, saponins, terpenoids, phenolics and flavonoids were present in both the extracts. In various tests for different categories of flavonoids, FE showed good presence of diverse flavonoids.
### Table 1

Phytochemical screening of extracts

| Phytochemical constituents | Aqueous extract | Fermented extract | Result |
|----------------------------|-----------------|-------------------|--------|
| **Alkaloids** (Dragendorff’s test) | +               | +                 | Brown ppt indicating Alkaloids |
| **Carbohydrates** (Fehlings test) | +               | ++                | Brown colour indicating sugars |
| **Saponins** (Foam Test)           | +               | ++                | Persistence of foam indicating saponins |
| **Steroids and Triterpenoids** (Salkowski test) | -               | -                 | Negative reaction indicating the absence of steroids |
| (Liebermann - Burchard Reaction)   | +++             | +++               | Green colour indicates Terpenoids |
| **Phenolic compounds & Tannins** (Ferric chloride test) | ++              | +++               | Dark bluish-green colour indicating condensed as well as hydrolyzable tannins |
| Lead acetate test                 | ++              | +++               | Reddish-brown ppt indicates tannins |
| **Flavone & Flavonoids** Sodium Hydroxide test | ++              | ++                | Intense yellow colour indicating flavonoids |
| Shinoda test                      | +               | ++                | Orange-red colour indicating flavanones & flavonols |
| Pacheco test                      | +               | +++               | Red colour indicates dihydroflavonols |
| Pew’s test                         | +               | +++               | Purple brown colour indicating dihydroflavonols & Flavanones |
| Antimony pentachloride test       | +               | +                 | Violet colour indicating chalcones |
| Vanillin hydrochloride test       | +++             | +                 | Pink colour indicates flavonoid glycosides |

**Table 1:** Phytochemical screening of Extracts

### 3.2 Physicochemical characterization during fermentation (Fermentation Kinetic Profile): In the study fermentation affected physical as well as chemical changes in the substrate dispersion. The physicochemical parameters can give an idea about the sequence of fermentation without any tedious analytical processing, *e.g.*, the alcohol content ensures that the fermentation has taken place. The other
tests like pH, solid content, and specific gravity indicate the physical nature of the extract. Throughout the fermentation process, pH, specific gravity, optical density, phenolic content, reducing sugar and alcohol production were measured at every 6 h up to 72 h. Table 2 shows the changes in various parameters as the time of fermentation progresses.
Table 2
Physicochemical changes during fermentation process

| Time (in h) | pH     | Sp. gravity | Optical density | TPC (mgGAE/100 g) | TFC (Quercetin equivalent) | Alcohol (%) | Red. Sugar (%) |
|------------|--------|-------------|-----------------|-------------------|----------------------------|-------------|----------------|
| 0          | 4.6±   | 1.059±0.05 | 1.213±0.03      | 231.65±3.2        | 102.55±2.1                 | 0.0±0.0     | 24.7±0.54     |
| 6          | 3.9±   | 1.076±0.02 | 1.532±0.02      | 315.82±4.2        | 135.48±3.2                 | 0.195±0.005| 28.4±0.51     |
| 12         | 3.7±   | 1.091±0.03 | 1.620±0.05      | 443.68±5.3        | 177.33±1.7                 | 0.526±0.02 | 24.4±0.60     |
| 18         | 3.3±   | 1.062±0.02 | 1.720±0.07      | 648.75±6.6        | 201.54±4.3                 | 1.051±0.019| 21.3±0.32     |
| 24         | 3.2±   | 1.054±0.05 | 1.800±0.05      | 833.23±8.2        | 227.82±2.6                 | 2.953±0.03 | 18.3±0.61     |
| 30         | 3.2±   | 1.064±0.03 | 1.850±0.06      | 1236.77±7.2       | 258.32±3.2                 | 4.165±0.04 | 14.6±0.45     |
| 36         | 3.1±   | 1.054±0.04 | 1.920±0.04      | 1540.54±10.6      | 278.25±5.2                 | 5.542±0.02 | 12.9±0.53     |
| 42         | 3.3±   | 1.060±0.03 | 2.050±0.03      | 1528.65±11.6      | 292.92±3.5                 | 6.023±0.04 | 10.6±0.33     |
| 48         | 3.1±   | 1.051±0.02 | 2.105±0.06      | 1485.46±8.4       | 321.64±1.7                 | 6.458±0.03 | 9.2±0.21      |
| 54         | 3.1±   | 1.050±0.05 | 2.321±0.05      | 1468.55±9.5       | 351.56±4.4                 | 8.312±0.03 | 7.8±0.23      |
| 60         | 3.15±  | 1.052±0.03 | 2.625±0.07      | 1443.64±11.4      | 362.75±5.7                 | 10.161±0.05| 6.7±0.12      |

The physicochemical parameters were evaluated every 6 hr to understand the changes affected by microbial action. Optical density rises throughout the experiment showing the long log phase of microbial culture which might be due to favorable conditions. Till 36 h, TPC rise exponentially whereas TFC rose upto 66 h. The maximum ethanol content was at 66 h. The maximum ethanol content was at 66 h (12.31%).
| Time (in h) | pH       | Sp. gravity | Optical density | TPC (mgGAE/100 g) | TFC (Quercetin equivalent) | Alcohol (%) | Red. Sugar (%) |
|------------|----------|-------------|----------------|-------------------|---------------------------|-------------|----------------|
| 66         | 3.2±0.03 | 1.052±0.03  | 2.780±0.06     | 1397.72±8.7       | 371.95±3.9                | 12.31±0.04  | 4.7±0.10       |
| 0.03       | 0.002    |             |                |                   |                           |             |                |
| 72         | 3.1±0.04 | 1.051±0.04  | 2.950±0.04     | 1358.88±12.4      | 370.42±4.7                | 12.22±0.03  | 4.4±0.13       |

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Table 2: **Physicochemical changes during fermentation process**

During fermentation, specific gravity decreases due to ethanol generation and utilization of free sugar content. pH becomes acidic due to the liberation of free acids. The optical density increases throughout the study period indicating exponential growth of the microbial culture. Also, the strain produced a good quantity of ethanol by consuming a major part of sugar content. *W. anomalus* is an ethanol tolerant yeast and can grow under high ethanol content. The content of phenolics shows great enhancement as well as the contents of flavonoids. Total phenolic content increases by 5.86 times and total flavonoid content increases by 3.61 times during fermentation (Fig. 1).

**Figure 1:** *Physicochemical changes as a function of time*

### 3.3 HPTLC Fingerprinting

The study employed 3 fruit materials that are rich in complex phenolics which yielded simple phenolics upon fermentative biotransformation thereby giving higher total phenolic content. Also, it has good flavonoid content along with flavonoid glycosides which gets hydrolyzed during the study yielding free flavonoid aglycones, estimated as Quercetin equivalents. The structures of various flavonoid compounds analyzed in the study are shown in Fig. 2.

**Figure 2:** *Structures of flavonoid compounds analyzed in the study*

**Figure 3:** *Comparative High Performance Thin Layer Chromatographic (HPTLC) fingerprinting of non-fermented and fermented extract in 366 nm and 254 nm*

Figure 3 shows the changes in the HPTLC profile of extract before and after fermentation. It shows TLC scanned under 366 nm and 254 nm respectively. Track nos. 1–3 are triplicate samples of NFE and track no. 4–6 are triplicate samples of FE. As is evident, the intensity of flavonoid spots in the upper portion of chromatogram increases after fermentation. It might be due to the hydrolysis of flavonoid glycosides.
present in source plant materials. Microbial biotransformation can be an easy way to efficiently convert glycoside to aglycones. Figure 4 shows the HPTLC densitogram for NFE and FE.

Figure 4: High Performance Thin Layer Chromatographic (HPTLC) densitogram for (a) Non fermented extract (b fermented extract)

Table 3 shows the quantitation of various compounds in NFE and FE. The quantitation was performed by the preparation of calibration curves. All 8 compounds showed good linearity under selected analytical conditions. The quantitation also corroborates the hydrolysis process during fermentation. The content of free aglycones increased in FE.

| S.No. | Compounds     | \( R^2 \) Value | Equation          | Content Before fermentation (mean ± SD, mg/ml) | Content After fermentation (mean ± SD, mg/ml) |
|-------|---------------|-----------------|-------------------|-----------------------------------------------|-----------------------------------------------|
| 1     | Apigenin      | 0.9995          | \( y = 94.827x + 75.616 \) | 3.37 ± 0.23                                   | 9.43 ± 31                                     |
| 2     | Luteolin      | 0.9991          | \( y = 107.14x + 314.95 \) | 2.44 ± 0.18                                   | 8.54 ± 0.27                                   |
| 3     | Quercetin     | 0.9988          | \( y = 68.515x + 533.99 \) | 6.28 ± 0.31                                   | 10.23 ± 0.22                                 |
| 4     | Kaempferol    | 0.9997          | \( y = 57.83x + 444.73 \) | 5.67 ± 0.25                                   | 9.65 ± 0.31                                  |
| 5     | Isorhamnetin  | 0.9989          | \( y = 40.842x + 297.57 \) | 4.77 ± 0.11                                   | 8.84 ± 0.42                                  |
| 6     | Hesperidin    | 0.9989          | \( y = 12.291x + 35.747 \) | 8.32 ± 0.32                                   | 2.82 ± 0.10                                  |
| 7     | Mangiferin    | 0.9997          | \( y = 11.46x + 33.333 \) | 5.32 ± 0.20                                   | 4.42 ± 0.21                                  |
| 8     | Corilagin     | 0.9990          | \( y = 10.057x + 187.33 \) | 6.44 ± 0.31                                   | 4.52 ± 0.2                                   |

The values are represented as Mean ± S.D., The no. of observations were 3.

Validation was performed to establish the validity of the method. All 8 compounds were quantitated in non-fermented and fermented extract.
Table 3: Linear regression data for calibration curves and contents of compounds before and after fermentation

1. **3.4 Acute Toxicity study**: All Extracts (NFE and FE) were evaluated for in vivo toxicity. Both the groups (lower & higher level) showed no behavioral or physical changes.

2. **3.5 Oral Glucose Tolerance test**: The rats were initially evaluated for normal pancreas function using the Oral glucose tolerance test. Table 4 showed the blood glucose level during the oral glucose tolerance test. FE showed good control over glucose levels at a lower dose level.

| Group               | Blood Glucose level |
|---------------------|---------------------|
|                     | 0 min       | 30 min       | 60 min       | 120 min      | 180 min      |
| Normal control      | 87.2 ± 1.5*  | 134.6 ± 1.2  | 121.4 ± 2.3**| 101.6 ± 0.9  | 91.1 ± 1.1*  |
| NFE (400 mg/Kg)     | 90.1 ± 1.6*  | 128.1 ± 1.1  | 116.1 ± 1.4* | 102.3 ± 1.6* | 96.4 ± 1.7   |
| FE (200 mg/Kg)      | 91.7 ± 1.3*  | 115.4 ± 1.4* | 99.3 ± 1.9** | 89.9 ± 1.2** | 80.6 ± 1.5** |
| Glibenclamide; 0.5 mg/Kg | 89.5 ± 1.4** | 112.2 ± 2.1* | 98.5 ± 1.5   | 90.6 ± 2.4   | 79.2 ± 2.1   |

The data is expressed as mean ± S.D. The observations were taken on 6 animals. The values having *p < 0.005 were significant and **p < 0.001 were highly significant.

Table 4: Blood glucose levels during Oral Glucose Tolerance Test

3.6 Antidiabetic activity

Antidiabetic activity was determined in Streptozotocin-induced diabetes of rats. As indicated by Table 5, FE restores the normal glucose level within 2–3 h. Also, the overall effect of 200 mg/Kg dose is similar to 400 mg/Kg. Based on correlation, free flavonoid content must be responsible for increased antidiabetic activity. Free aglycones are less bulky and more lipophilic thereby easy to absorb through the small intestine. This increases the effective bioavailability and in turn, increases the antidiabetic activity.
Table 5
Blood glucose levels during Antidiabetic activity

| Group | Treatment (mg/kg b.w.) | Blood glucose (mg/dl) |
|-------|------------------------|-----------------------|
|       |                        | Day 0  | Day 7  | Day 14 | Day 21 | Day 28 |
| I     | Normal saline (0.5 ml/kg) | 91.54 ± 1.1  | 91.32 ± 1.9** | 91.69 ± 0.8** | 90.90 ± 0.9* | 91.00 ± 0.7* |
| II    | Normal saline + STZ (0.5 ml/kg + 60 mg/kg) | 290.0 ± 1.5* | 311.0 ± 1.4 | 327.8 ± 1.7* | 332.9 ± 2.2 | 345.8 ± 1.9* |
| III   | NFE (400 mg/kg) + STZ | 323.0 ± 1.5* | 295.8 ± 1.2* | 233.3 ± 2.1* | 175.0 ± 2.4* | 135.0 ± 1.4** |
| IV    | FE (200 mg/kg) + STZ | 338.6 ± 1.2** | 164.4 ± 1.5** | 107.4 ± 1.5** | 89.8 ± 1.7** | 90.8 ± 1.2** |
| V     | Glibenclamide (0.5 mg/kg) + STZ | 310.2 ± 1.5* | 239.1 ± 1.3* | 189.3 ± 2.4* | 116.5 ± 1.1* | 111.7 ± 2.1* |

All values were expressed as mean ± SD (n = 6). *p < 0.005 when compared to control group **p < 0.001 when compared to standard (One way ANOVA followed by Dunnett’s test).

Glibenclamide showed a gradual decrease in 21 days, the effects plateau further. Non-fermented extract showed a similar pattern till 28 days whereas fermented extract showed almost similar reduction in blood glucose within 7 days.

Table 5: Blood glucose levels during Antidiabetic activity (OGTT)

3.7. Histopathological studies:

Figure 5 shows the histological structures of Kidney, pancreas and liver of Normal, diabetic and treated (Non-fermented ex. & fermented ex.) animals. As is evident, the fermented as well as non-fermented extract showed a great extent of restoration in vital organs. However, it is pertinent to note that fermented extract has been more effective in the restoration of normal histological infrastructure.

Figure 5: Histopathological comparison of normal, diabetic, Fermented extract-treated and Non-fermented extract-treated animals

4. Conclusion

The present study summarizes the physical, chromatographic and therapeutic transformation in polyherbal extract upon fermentation. W. anomalus, being an ethanol-producing strain, generates a substantial quantity of ethanol thereby decreasing the specific gravity, however, the microbial strain also affected the pH of the extract. Further, the strain grows steadily as indicated by enhanced optical density. The effects on chemical profile initially reflected an increase in TPC and TFC. In chromatographic analysis of NFE and FE, the main difference was the content of aglycones. In NFE, several glycosides were present but after fermentation, the content of glycosides decreased indicating the occurrence of
glycolysis and hydrolysis during fermentation. During fermentation, various acids are released, decreasing the pH and catalyzing the acidic hydrolysis of glycosidic bonds. Most oligomeric flavonoids are converted to monomers and dimers under acidic conditions. Further, *W. anomalus* can produce glycosidases which can directly attack the glycosidic bonds to release the aglycones. Based on physicochemical and chromatographic observations, it can be said that the microbial cells grew and feed off the free sugar to generate ethanol however to maintain the osmotic stress, glycosides are hydrolyzed and free sugars generated. The fermentation makes the chemical composition of FE more lipophilic and free aglycones are directly absorbed by the small intestine. The pharmacological effects are further corroborated by histological observations. The enhanced content of aglycones might be the main reason behind the enhanced antidiabetic activity of FE.

**Declarations**

**Acknowledgment**

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**Conflict of Interest:** None

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**Figures**
Physicochemical changes as a function of time (A) Biomass was determined in terms of optical density, as the biomass increases, it shows turbidity in culture medium resulting in higher optical density. (B) The reducing sugar content decreases and ethanol content increases as time progress. Ethanol content increases till 66 h, the highest being 12.31 %. (C) TPC was estimated in terms of mg Gallic acid equivalents and TPC was exponentially increases till 36 h. (D) TFC was determined as quercetin equivalent and was continuously increased till 66 h. It indicates the occurrence of hydrolysis and de-glycosylation respectively.
Figure 2
Structures of flavonoid compounds analyzed in the study Apigenin and Luteolin belong to Flavone category, quercetin, kaempferol and Isorhamnetin belong to flavonols. Mangiferin is a xanthone glycoside, hesperidin is flavanone glycoside, Corilagin is phenolic glycoside.

**Figure 3**

Comparative High Performance Thin Layer Chromatographic (HPTLC) fingerprinting of non-fermented and fermented extract in 366 nm and 254 nm Track nos. 1, 2 and 3 are non-fermented extracts and 4, 5 and 6 are fermented extract showing the presence of polar compounds in non-fermented extract and non-polar compounds in fermented extract.
Figure 4

High Performance Thin Layer Chromatographic (HPTLC) densitogram for (a) Non fermented extract (b) fermented extract. Densitograms show polar compounds in non-fermented extract and non-polar compounds in fermented extract.
|                      | Kidney                                      | Pancreas                                   | Liver                                      |
|----------------------|---------------------------------------------|--------------------------------------------|--------------------------------------------|
| **NORMAL**           | ![Normal Kidney](image1)                    | ![Normal Pancreas](image2)                | ![Normal Liver](image3)                   |
|                      | Normal tubular network and glomerular       | Normal pancreatic islets and architecture. | Normal hepatocytes and sinusoidal plexuses. |
|                      | architecture                               |                                            | Mild inflammation and absence of necrosis  |
| **DIABETIC**         | ![Diabetic Kidney](image4)                  | ![Degenerative Pancreas](image5)          | ![Cohesive Necrosis](image6)               |
|                      | Necrosis, vacuolar degeneration, dilatation |                                            |                                            |
|                      | of tubules, cell infiltration               |                                            |                                            |
| **FERMENTED**        | ![Fermented Kidney](image7)                 | ![Normal Pancreas](image8)                | ![Regenerated Liver](image9)              |
|                      | Normal tubular diameter, glomerular         | ![Normal Architecture](image10)           |                                            |
|                      | regeneration                               |                                            |                                            |
| **NON-FERMENTED**    | ![Non-Fermented Kidney](image11)            | ![Few Areas of Inflammation](image12)     | ![Sinus Under Regeneration](image13)      |
|                      | Normal architecture with slight damage      |                                            |                                            |
|                      | Regenerated sinusoidal network, Normal      |                                            |                                            |
|                      | hepatocytes                                |                                            |                                            |

**Figure 5**

Histopathological comparison of normal, diabetic, Fermented extract-treated animals and Non-fermented extract-treated animals.